Radial Glia and Neurogenesis in the Embryonic Zebrafish Hindbrain

Adam Tsuda Guy

Submitted to the University of London in 2004 in partial fulfilment of the requirements for the award of

PhD

University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

This thesis is an investigation of the behaviour of radial progenitors in the hindbrain of the teleost fish *Danio rerio*, the zebrafish, during embryonic neurogenesis. These progenitors have many of the characteristics of radial glial cells described in other systems. Recent studies from several laboratories worldwide have shown that radial glia play an important role in the patterning and neurogenesis of the mammalian forebrain. However, despite the enormous interest in the subject, investigations into neurogenic radial glia have to date been largely limited to the mouse telencephalon. No similar study has yet investigated embryonic neurogenic radial glia in other vertebrates, and very few have been carried out in brain regions other than the forebrain. In this study I used the zebrafish as a model system to identify and characterise radial glia and progenitors in the hindbrain, and examine the role of these cells in neurogenesis in this brain region. Radial progenitor cells in the hindbrain ventricular zone (VZ) were found to express glial fibrillary acidic protein (GFAP) during embryonic development, and some of these cells also expressed markers of proliferation such as PH3. Fatemapping these radial glia-like cells using single-cell labelling techniques, I demonstrated that the major neurogenic event in the zebrafish hindbrain after 48hpf is direct differentiation of progenitors into neurons, without cell division. Very few cell divisions were recorded, and those few that were observed were without exception symmetric, neuron-pair terminal mitoses. In contrast to previous studies of mammalian neurogenesis, no asymmetric cell divisions were observed in my experiments. I then examined the behaviour of VZ progenitors as a population, by taking advantage of the Tg(zFoxD3:GFP) stable transgenic line. In this line, a subpopulation of hindbrain VZ progenitors expresses GFP during development, and I recorded timelapse movies of hindbrain neurogenesis, in transgenic embryos, between 34hpf and 50hpf. This revealed the radial migration of GFP-expressing newborn neurons from VZ into the mantle layer, and how these cells utilise multiple GFAP-expressing radial processes as a substrate for this migration. These timelapse videos are the first description of radial migration in a vertebrate brain *in vivo*.

Acknowledgments

I must begin by thanking my PhD supervisor Jon Clarke, who took the most unusual step of accepting me as a student, despite the fact that as a pharmacologist I knew nothing about development, knowledge which one might consider useful when studying for a PhD in developmental neurobiology. His patient supervision and tolerance of my various peculiarities have contributed much to making this thesis possible. Similarly, I must thank the rest of the Clarke lab. I am gravely indebted to David Lyons, Philippa Bayley and Marcel Tawk, who between them have provided eating competitions, the many now-legendary drinking sessions and diverse other amusements, in addition to teaching me almost all of what I know about zebrafish development and microscopy. The laboratories of Paul Martin, John Scholes and Steve Wilson too provided much in the manner of bibulous company and other refreshing entertainments. Before arriving at UCL many kind people have guided me as I stumbled, tired and confused, towards realising my goal, to whom I owe many thanks. David Hudson and John Masters, for entertaining, with uncommon good humour, my clumsy attempts at scientific research as an undergraduate placement student many years ago; and Peter Whitton, with whom the manifold discussions of association football managed to obscure the various failings of my B.Sc. research project. Special mention too must be made of both Hans Schürch and the Courage Brewery, whose products have unfailingly provided me with much solace during those dark moments when everything seemed so difficult. Finally, above all else, I must thank my mum and dad, whose support has been endless and unconditional. I can only thank the powers that be, for being so lucky to be in the position I am now.

Table of contents

Chapter One

List of Figures, Graphs and Tables

Chapter Six: General Discussion

Supplementary Movies

These movies are all on a CD-ROM that accompanies this thesis. They are saved in Quicktime (QT) format and can be viewed in Quicktime, NIH Image or Openlab.

Chapter Five: Analysis of Hindbrain Neurogenesis in the Stable Transgenic Tg(zFoxD3:GFP) Line

Movie One: Timelapse imaged from 36hpf to 48hpf. Lateral view, anterior is to the left. Images were captured every three minutes.

Movie Two: As above, from 32hpf to 47hpf. This movie was recorded at a more lateral focal plane, hence the appearance of fewer GFP-expressing cells.

Movie Three: As above, but the timelapse was recorded from a later start point, from 38hpf to 54hpf.

Chapter One

General Introduction

The adult human brain is the most complex organ in the body and is comprised of numerous distinct cell types performing a multitude of specialised functions, only a part of which are fully understood. However, the brain is initially derived from an apparently uniform population of progenitors within the neuroectoderm very early in development. The progression from a homogeneous population of progenitors arranged in a single sheet to the complex and highly organised structure of the adult brain is a process that lends itself to almost limitless investigation. One fundamental question is how does the embryonic brain generate enough cells required for correct functionality in the adult human brain? Related to this point is another crucial issue, how the progenitor cells of the primitive brain create the necessary different cell types required from this original, homogeneous population. The cells that comprise the central nervous system are divided into two main types, neurons and glia. The dynamics of vertebrate neurogenesis – how progenitors give rise to the neurons and glia to populate the brain – is a topic of great scientific interest yet there remain many gaps in our knowledge of these processes. For example, recent findings have indicated that the neuronal and glial lineages are not as separate as previously thought, and that some glial cells can generate neurons and may even act as neural stem cells.

In this thesis I use the zebrafish as a model organism to make novel observations of neurogenesis in vivo during late embryonic development. In particular, using live imaging, cell tracing techniques and transgenic embryos the behaviour – cell divisions, differentiation and migration – of CNS progenitors will be examined, both at the level of the single cell and as a population. Recent research in the laboratory has challenged many of our long-held conceptions of lineage and neurogenesis. There is a clear need for further insight into the nature of the enigmatic radial glia, particularly in submammalian vertebrates. Radial glial cells in the zebrafish CNS will be identified and characterised, and their contribution to neurogenesis analysed.

Definitions Used in This Thesis

In the course of this thesis I will be using much terminology devoted to lineage, division symmetry and phenotype. Therefore before continuing, for the sake of consistency and to avoid confusion it is necessary to define some of these terms that will be used, since like any subject, many researchers have differing interpretations of the same terminology. Therefore, in the context of this thesis,

- *Asymmetric cell division* is one where the developmental fate of the two daughter cells differs. This definition does not imply a mechanism such as the balance of intrinsic fate determinants, rather, it rests solely upon the phenotype of a cell being either progenitor or neuronal.
- *Symmetric cell division* is a division where the phenotype of the two daughter cells is the same. In the context of this study, this refers to progenitor-pair or neuron-pair cell divisions.
- *Neuroepithelial* cells are the undifferentiated, or most primitive, progenitor of the CNS. These cells are recognisable by their bipolar morphology with endfeet contacting both apical (ventricular) and pial (basal) surfaces, and their often ovoid or slender cell body. As neurogenesis progresses the somata of these cells become restricted to the ventricular zone (VZ) and as they proceed through the cell cycle, undergo interkinetic nuclear migration.
- *Stem cell* will only be used to describe cells that fulfil the three classical criteria of asymmetric cell division, self-renewal and immortality. Therefore transit-amplifying cells are distinguishable from stem cells in their limited lifespan, although some may be capable of self-renewal. By these criteria most mammalian radial glia do not qualify as stem cells since they are not immortal, although radial glia in many non-mammalian vertebrates, such as those found in *Ambystoma*, probably are.
- *Progenitor* will be the term used to describe any cell that gives rise to a more differentiated cell type. All stem cells are progenitors, yet the reverse is not true, since by this definition 'progenitor' includes transit-amplifying cells that divide symmetrically to expand their numbers and then divide to produce two neurons, or a precursor that directly differentiates without cell division, for example.

- *Lineage study* is a term that will be applied only to an experiment where the lineage history or tree of single cells can be unambiguously reconstructed from the point of cell labelling till the conclusion of the experiment.
- *Clonal analysis* refers to the experimental technique of labelling single cells and observing the resulting clone after a certain period of time has elapsed. This technique reveals some of the developmental potential of cells, but cannot determine their lineage, if the clone contains three or more cells.

The Ventricular Zone

Composition of the Ventricular Zone

Ever since the first description of progenitors in the vertebrate nervous system by Wilhelm His, the germinal or ventricular zone of the neural tube has been the subject of great study and fascination (His, 1887). Sauer first demonstrated that the various morphologically diverse cells identifiable in the pseudostratified epithelium that comprises the early neural tube were in fact neuroepithelial cells in different stages of the cell cycle (Sauer, 1935a and 1935b). As development progresses, the cells of the neural tube become divided into ventricular zone (VZ) which is the undifferentiated, proliferative compartment of the brain, and the mantle layer or mantle zone (MZ) which consists of differentiated, post-mitotic neurons. Since historically it has not been possible to observe these cells alive *in situ* – in mammals at least – in a timelapse manner, the behaviour of different cells in the developing VZ was largely hypothesised from observations of fixed tissue. For example, what physically happens at the point when a progenitor divides is often taken for granted. The textbook view is that neuroepithelial cells lose or retract their basal and apical processes and round up at the ventricular surface when undergoing cell division in M-phase; this behaviour was reported as part of the cell cycle by Sauer, and had apparently been corroborated by Golgi and electron microscopy surveys (Sauer, 1935b, Hinds and Ruffet, 1971 and Seymour and Berry, 1975). However, in the last few years timelapse microscopy studies have shown that when a progenitor cell rounds up at the ventricular surface during cell division, it often retains a fine basal process in contact with the pia (Noctor et al., 2001 and 2004, Miyata et al., 2001, Das et al., 2003 and D. Lyons, personal communication). Miyata and colleagues explain that it is probably only with live-imaging that this

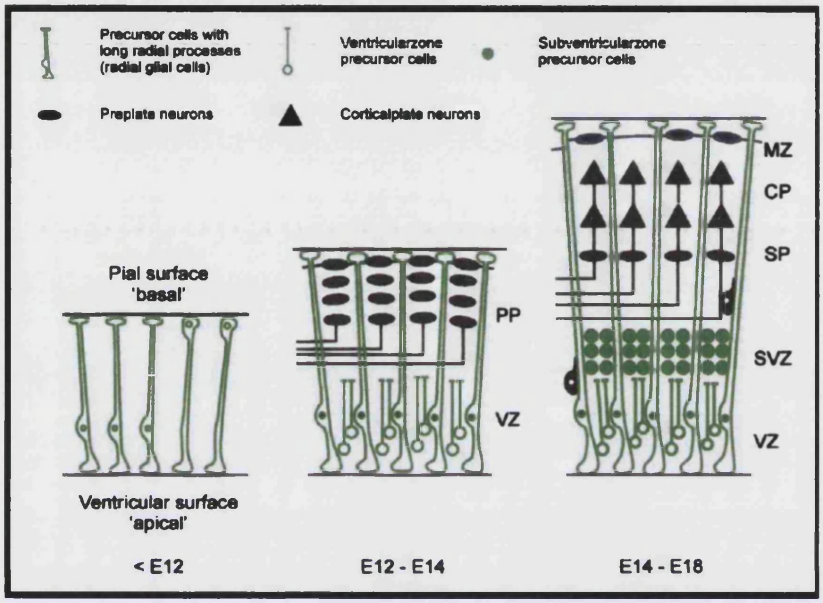
maintenance of pial contact is apparent, since the process is very fine (<0.5μm) and probably becomes lost or indistinct following methods of fixation, especially with gluteraldehyde, that cause tissue shrinkage (Miyata et al., 2001).

The cellular composition of the VZ, and how the population changes during development, is also not entirely clear. It is well accepted that early in development, the neural tube is composed largely of neuroepithelial cells. Neuroepithelial cells are the most undifferentiated and primitive cell type of the brain, the progenitors that ultimately give rise to all the differentiated cells of the adult CNS. They are bipolar, radial cells with processes spanning the apicobasal dimension and contacting the ventricular and pial surfaces. What happens as neurogenesis proceeds however, is not well known, although the widely accepted model, until recently, is summarised in Figure 1.1. It is believed that as the neural tube expands and becomes more complex, neuroepithelial cells lose their contact with the pia, and extend their basal processes only as far as the basal extent of the VZ, becoming cells whose entirety is resident in the VZ only (Figure 1.1). These 'shortened' neuroepithelial progenitors divide and their progeny migrate radially to the mantle zone, using radial glial processes as a guide to direct this movement (Figure 1.1). According to this traditional model, the only cells that remain in contact with both ventricle and pia in mid- to late neurogenesis stages are radial glia. Because of this view, it was thought that radial glial cells were relatively quiescent and did not divide, since rounding up of their cell body during mitosis would mean they would lose their neuron-guiding radial process (Schmechel and Rakic, 1979b).

Another resident of the embryonic VZ, albeit a temporary one, is the nascent or newborn neuron. Newborn neurons express markers such as NeuroD and Notch ligands (see Lewis, 1998 and Jan and Jan, 1994) but do not possess complete neuronal morphology such as dendrites and axonal connections. Since M-phase of the cell cycle occurs at the ventricular surface, newborn neurons are required to migrate out of the VZ and into more basal positions in the MZ to reach their appropriate final position. How this migration may take place is described in further detail later, but the important point to bear in mind is that observation of fixed tissue alone can sometimes give the false impression of the VZ being a static or stable zone, whilst in reality the VZ is a somewhat turbulent environment, with continuous cell movement and migration taking place within this compartment. In addition to many mitoses at the ventricular surface, there is

Figure 1.1. Proposed model of corticogenesis

Adapted from Götz et al, 2002. Progenitor cells are indicated in green, and postmitotic cells in black. Prior to neurogenesis (<E12), the neural tube consists entirely of precursors with long, radial processes. These cells are bipolar and radial in character, and display the characteristic 'elevator movement' of interkinetic nuclear migration as they go proceed through the cell cycle. During early neurogenesis (E12 – E14) the earliest-born neurons give rise to the preplate (PP). Radial glia are evident as they maintain their long radial basal process in contact with the pial surface, whilst the majority of progenitors within the ventricular zone (VZ) taken on a 'shortened' morphology. The basal processes of these cells terminate within the VZ. From E14 onwards (E14 – E18) the characteristic layers develop and the cortex takes on a more complex histology. During this period a second compartment of progenitors forms, the subventricular zone (SVZ). Neurons born in the VZ migrate along the radial processes of radial glia to reach the cortical plate (CP). According to Götz and colleagues, by this model the only cells to maintain contact with the pial surface during neurogenesis are radial glia.


Precursor cells with long radial processes (radial glial cells)
Ventricularzone precursor cells
Subventricularzone precursor cells
Preplate neurons
Corticalplate neurons
Pial surface 'basal'
Ventricular surface 'apical'
PP
VZ
MZ
CP
SP
SVZ
VZ
< E12
E12 - E14
E14 - E18

the constant 'elevator movement' or interkinetic nuclear migration of cycling neuroepithelial progenitors and newborn neurons migrating out of the VZ to reach their correct apicobasal position.

In addition to neuroepithelial cells, another major cell type in the ventricular zone during neurogenesis is the radial glial cell. Radial glia as a cell type was first reported well over a century ago and its history is complex (reviewed by Bentivoglio and Mazzarello, 1999 and Rakic, 2003). Using the heavy metal impregnation technique pioneered by Italian histologist Camillo Golgi, a number of eminent researchers such as Wilhelm His, Gustaf Retzius, Rudolf Albert von Kölliker and Golgi himself reported in preparations of the foetal cortex of various mammals such as rabbit or calf, numerous cell somata in the ventricular zone that possessed a long fine process extending to the pial surface (Figure 1.2). Italian embryologist Giuseppe Magini named these cells 'radial neuroglia' in 1888 (Magini, 1888, in Bentivoglio and Mazzarello, 1999). Magini also noted that in his observations of foetal calf cortex it appeared that other cell bodies were closely associated with some of the radial processes belonging to his radial neuroglia; he hypothesised that these were cells that were in some way communicating with the radial process, a theory that was contested by many of his contemporaries such as Santiago Ramón y Cajal, who insisted that they were not separate cells but 'varicosities' or swellings in the cell membrane of the radial process (in Bentivoglio and Mazzarello, 1999). It took over eighty years and the advent of the technology of electron microscopy to vindicate Magini's view, for in 1971 Pasko Rakic reported that newborn neurons in the foetal macaque cortex appeared to be migrating along the radial processes of radial glia; his conclusion was that radial glia create a scaffold of processes that guide neurons to their appropriate apico-basal target and he dubbed these cells 'radial glia' (Figure 1.2) (Rakic 1971a and 1972; for review, see Rakic, 1990 and 2003). Rakic hypothesised that unlike most cells of the glial lineage that are generated after the majority of neurons arise, many radial glia are apparently born just before neurogenesis, making them one of the first differentiated cell types in the mammalian CNS (Rakic, 1972). Later, other studies apparently confirm this early, pre-neurogenesis appearance of radial glia (Misson et al., 1988). Although they were first reported in mammals, radial glia can also be found in the developing CNS of all vertebrates: following the work of Magini, Golgi and von Kölliker, the Golgi method was used to label and

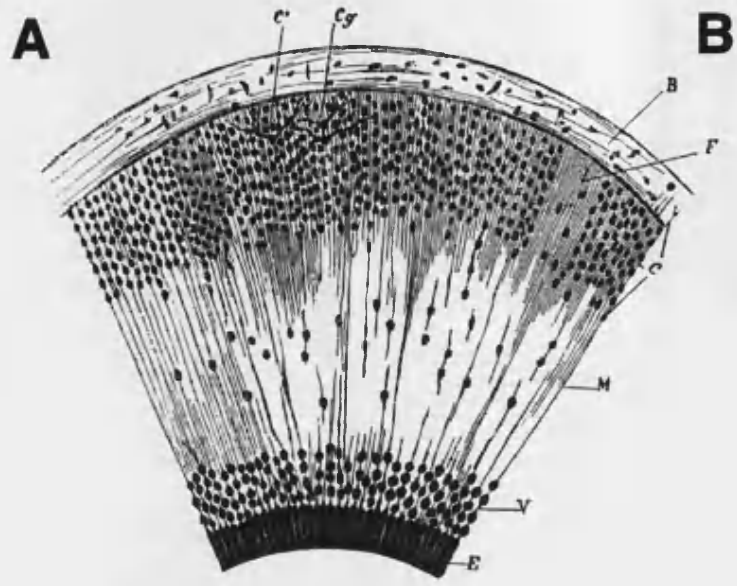
Figure 1.2. Identification of mammalian radial glia

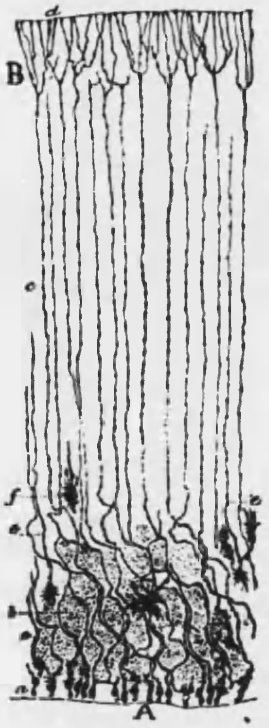
A. Using the Golgi technique, Guiseppe Magini first described 'radial nevroglia' in the foetal calf cortex (Magini, 1888). The somata of these cells lay in the ventricular zone (V) and possessed long, fine processes that extended radially, 'like the spokes of a wheel', toward the marginal zone (B). Adapted from Bentivolgio and Mazzarello, 1999.

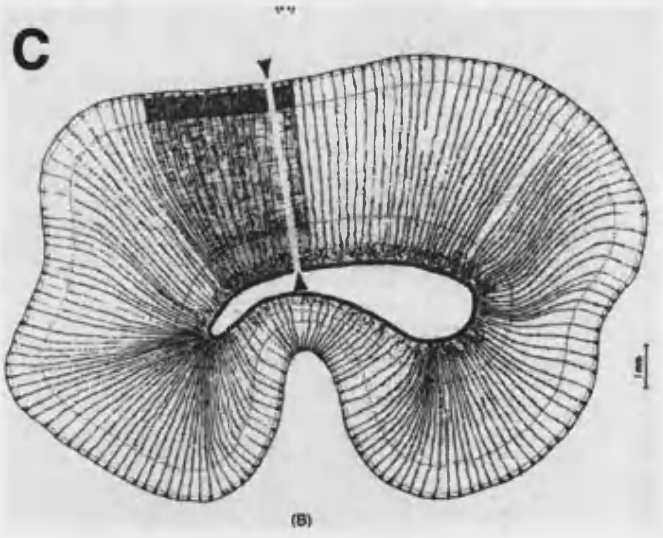
B. Santiago Ramón y Cajal's Golgi preparation of neonate rabbit cortex. The radial glia (a) lie in the ventricular zone, with either their cell body or characteristic club-like apical process in contact with the ventricular surface (A). After negotiating a tortuous route through the axons of the intermediate zone (b) their radial processes penetrate the cortical plate (c) and extend through to the pia (d). A notable observation by Ramón y Cajal is the characteristic branching of the radial glial processes once they reach the interface between cortical plate and marginal zone (B), yet most of the branches contact the pial surface. However, one *caveat* exists when perusing micrographs by Ramón y Cajal: rather than record exactly what he saw down the microscope, apparently he made idealised drawings representative of his own hypotheses of what was taking place in the tissue (see Jacobson, 1999). Adapted from Bentivoglio and Mazzarello, 1999.

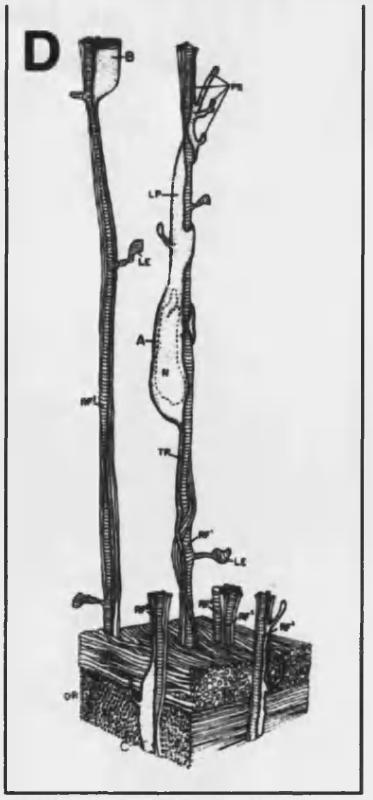
C. Low-magnification sketch of a Golgi-stained coronal section of E97 macaque cortex, showing the radial network of radial glial process, by Pasko Rakic (Rakic, 1971a, 1971b and 1972). Rakic's description of radial glia in the foetal macaque cortex had, until very recently, defined the field of radial glial biology. Using Golgi-stained preparations and serial electron microscopy, Rakic described the radial processes of radial glia as a scaffold or 'guiding rail' for the apicobasal migration of newborn neurons from ventricular zone to mantle layer. Adapted from Bentivoglio and Mazzarello, 1999.

D. Reconstructed diagram by Rakic, describing the intimate relationship between radially migrating newborn neurons (A, B and C) and radial fibres (RF). The migrating neuron 'A' extends a leading process (LP) along the radial fibre, and is tipped with a number of pseudopodial processes (PS). The neuron also possesses a trailing process (TP) that is very fine and also closely associated with the radial fibre. To the casual observer, the appearance is of the leading process 'dragging' the cell body, including the majority of the cytoplasm and the nucleus (N), along the radial fibre. Both radial fibres also show horizontally projecting lamellate extensions (LE). Rakic painstakingly reconstructed serial EM sections of Golgi-stained preparations to make these observations, therefore his hypotheses of radial migration were based purely on inference from fixed tissue. Over thirty years hence, still no researcher has visualised radial migration actually taking place, in an intact, living brain. Adapted from Rakic, 2003.


A
B
F
C
M
V
E


B
A


C
(B)


D

identify radial glia in jawless fish (Retzius, 1893), cartilaginous fish (von Lenhossék, 1892), bony fish (van Gehuchten, 1894) and amphibians (van Gehuchten, 1898) (Figure 1.3). Amazingly, the same method of silver impregnation pioneered by Camillo Golgi remains in use in modern times, with only minor modifications (e.g., the 'rapid' or 'mixed' methods such as Golgi-Cox or Golgi-Stensaas). Rakic used the technique to label radial glia in foetal macaque cortex (Rakic, 1971a) and cerebellum (Rakic, 1971b) and it has also been used to label such cells in marsupials (Morest, 1970), lizards (Garcia-Verdugo et al., 1986), toads (Stensaas and Stensaas, 1968a) and pigeon (Stensaas and Stensaas, 1968b) (Figure 1.3).

The Golgi method of staining does not specifically label any single cell type. The traditional molecular marker of radial glia is glial fibrillary acidic protein (GFAP). GFAP is an intermediate filament protein first isolated by Larry Eng and colleagues from pathological human fibrous astrocytes (Eng et al., 1971) and although it was originally used as a marker of astrocytes, GFAP is also expressed by radial glia in many vertebrates, with rodents being a curious exception (Bignami and Dahl, 1974, Sancho-Tello et al., 1995). However, despite being isolated over thirty years ago, the exact function of the GFAP protein remains a mystery, with attempts to understand it creating further confusion. Little is known about the function of GFAP other than it is an intermediate filament protein that probably confers upon reactive astrocytes their dynamic, multiple processes and connections; GFAP is highly upregulated in these cells during gliosis (Kumanishi et al., 1992, reviewed by Brenner et al., 1994 and Eng et al., 2000). The GFAP knockout mouse made independently in four different laboratories showed extremely subtle phenotypes (for review, see Messing and Brenner, 2003) when compared to the drastic human situation. In humans, missense point mutation in the GFAP gene leads to the fatal childhood leukodystrophy Alexander Disease, a severe neurodegenerative illness characterised by mental and developmental retardation, feeding and behavioural disorder, megaencephaly, hydrocephalus and seizures (reviewed by Li et al., 2002). The symptoms of the human disorder are currently attributed to a fibrosis of the brain, with histopathology revealing characteristic Rosenthal fibres, eosinophilic fibrous inclusion bodies in the cytoplasm of astrocytes. However, whilst mouse knockouts have consistently failed to reproduce this phenotype, the gain-of-function mouse, with a knock-in human GFAP insert producing 15-20 times

Figure 1.3. Vertebrate radial glia revealed by the Golgi method

A. van Gehuchten's Golgi preparation of adult trout forebrain revealed radial glial cells (van Gehuchten, 1894). His drawing clearly shows the ovoid or slender soma, radial processes contacting both ventricular and pial surfaces, process branching, endfeet and spiny elaborations characteristic of radial glia. From van Gehuchten, 1894.

B. Two preparations by the same author in salamander spinal cord (van Gehuchten, 1898). To the left, the radial glia in the section is labelled 'c'. He describes the interesting cell labelled 'd' as "cellule ependymaire ayant perdu toute connexion avec le canal central", speculating it had transformed from a morphology similar to that of cell 'c'. To right, another example, this time displaying more spiny elaborations of the radial processes and their characteristic rounded or club-shaped endfeet. Both radial glia appear to have a more irregular shape to their soma compared to the more ovoid and smooth forms described elsewhere. From van Gehuchten, 1898.

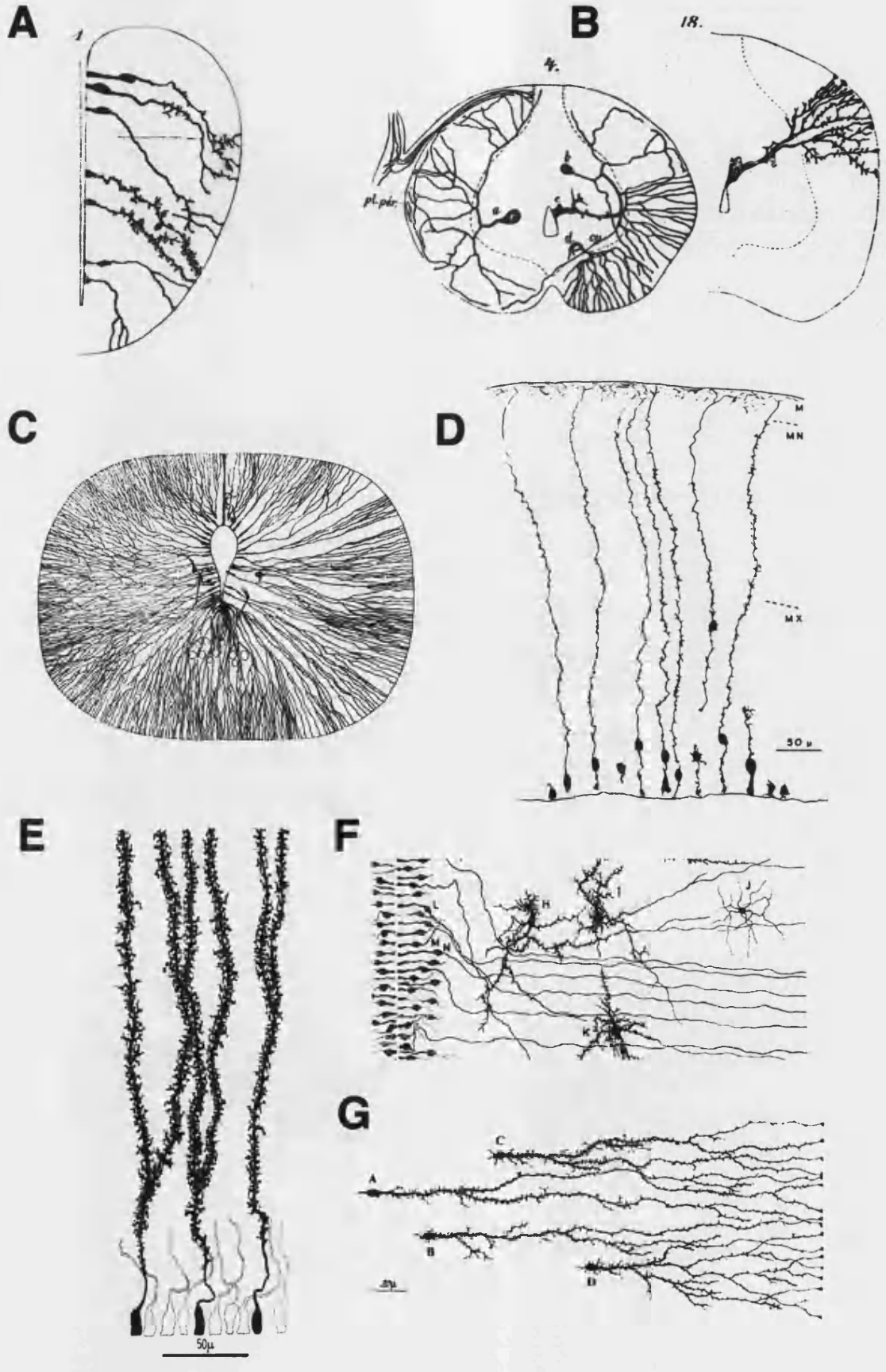
C. Gustaf Retzius labelled radial glial cells in the spinal cord of adult *Petromyzon*, demonstrating that such cells were also present in the CNS of aganthan vertebrates, in this case lamprey (Retzius, 1893). From Bentivoglio and Mazzarello, 1999.

D. Radial glia in the forebrain of pouch opossum (Morest, 1970). These cells do not show the extensive branching revealed in other studies, but possess the ovoid soma, ventricular and pial-contacting radial morphology and some moderate spiny excrescences noted by other workers. From Morest, 1970.

E. Morphology of somata and proximal processes in radial glia of the adult pigeon forebrain (Stensaas and Stensaas, 1968a). Here, the cell bodies are abutted closely with the ventricle, giving the cells a monopolar appearance. Of particular note is the extent of lamellate elaborations on the radial glial processes; the authors report that this is characteristic of radial glia '...in the cerebral cortex are covered by innumerable lamellate excrescences giving them a hirsuite appearance". From Stensaas and Stensaas, 1968a.

F. Morphology of radial glial somata in the adult turtle spinal cord, by the same authors. The spinal cord lumen is to the left, bordered by the cell bodies of radial glia ('L – N'). 'H', 'I' and 'K' are protoplasmic astrocytes and 'J' is apparently a labelled oligodendrocyte. Adapted from Stensaas and Stensaas, 1968a.

G. Radial glia in the spinal cord of adult toad *Bufo* (Stensaas and Stensaas, 1968b). Only cell 'A' is in contact with the ventricle, but all are within the grey matter. According to the authors, 'the density of fine excrescences is usually greatest within the gray matter, but it varies from one cell to the next. However, it is generally low in the peripheral plexus just beneath the surface where the major processes terminate in large end-feet.' From Stensaas and Stensaas, 1968b.


A
B
18.
4.
pl. pér.
C
D
M
MN
MX
30 µ
E
50µ
F
G

the constitutive levels of protein, exhibits the Rosenthal fibrosis and early fatality similar to Alexander Disease (Messing et al., 1998). It is yet unexplained why the overexpression of wildtype GFAP in mice, but not the truncated mutant protein itself, phenocopies the human situation. The GFAP gene has been cloned in several species including mouse, human and zebrafish (Cowan et al., 1985, Kumanishi et al., 1992 and Nielsen and Jorgensen, 2003) and a transgenic mouse in which astrocytes express GFP has also been raised (Nolte et al., 2001). There have also been a number of antibodies raised to the protein. Although first used in humans (Choi and Lapham, 1978), antisera to GFAP generally cross-react well between species, highlighting the high level of conservation between vertebrates, and has since been demonstrated to be expressed by radial glia in a variety of mammals such as macaque (Levitt and Rakic, 1980), cats and dogs (Onteniente et al., 1983) and also in birds, reptiles, amphibians and many species of bony and cartilaginous fish (Dahl and Bignami, 1973, Dahl et al., 1985, Onteniente et al., 1983, Kálmán 1998 and 2002, Kálmán and Gould, 2001, Ahboucha et al., 2003 and Arochena et al., 2004). Some examples of GFAP-expressing radial glia are shown in Figure 1.4.

In modern times other markers of radial glia have been described (Figure 1.5). However, most of these markers do not distinguish between radial glia and neuroepithelial VZ progenitor cells and generally label most of the cells in the VZ. Antibodies to the intermediate filament nestin, or *N*euro*E*pithelial *ST*em cell gene (Lendahl et al., 1990) and the marker 'radial cell-2' or RC2 (Misson et al., 1988), which is probably a modified form of nestin (Chanas-Sacré et al, 2000), label almost all progenitors in the VZ from E9. However, nestin is not a reliable marker as it is expressed in the developing muscle and teeth (Sejersen and Lendahl, 1993 and Terling et al., 1995). Most RC2-expressing progenitor cells in the cortical VZ also express Pax6, localised to their nuclei (Götz et al., 1998). The astrocytic glutamate transporter GluT-1 or EAAT-1, commonly known as GLAST, is also expressed by many VZ cells during early neurogenesis, and is co-expressed with nestin and RC2 (Shibata et al., 1997). Additionally brain lipid-binding protein (BLBP) has been described as a marker of VZ cells (Feng et al., 1994) and is expressed by many radial glia in the cortex (Hartfuss et al., 2001 and Anthony et al., 2004). According to the characterisation of cortical expression of these markers performed by Hartfuss and colleagues, from E12 through to

Figure 1.4. Vertebrate radial glia revealed by GFAP antibody staining
GFAP is expressed by radial glia in many vertebrates, including bony and cartilaginous fishes, reptiles and amphibians.
A. GFAP-expressing radial glial processes in the brainstem of *Chelon labrosus*, the grey mullet. Adapted from Arochena et al., 2004.
B. GFAP-expressing radial glial cell bodies and processes in the CNS of *Scyliorhinus canicula*, the small-spotted catshark. Adapted from Wasowicz et al., 1999.
C. Processes and radial glial cell bodies in the midbrain of *Tarentola mauritanica*, the crocodile gecko. Adapted from Ahboucha et al., 2003.
D. Immunofluorescence antibody stain to GFAP in the spinal cord of *Xenopus*. Adapted from Yoshida, 2001.

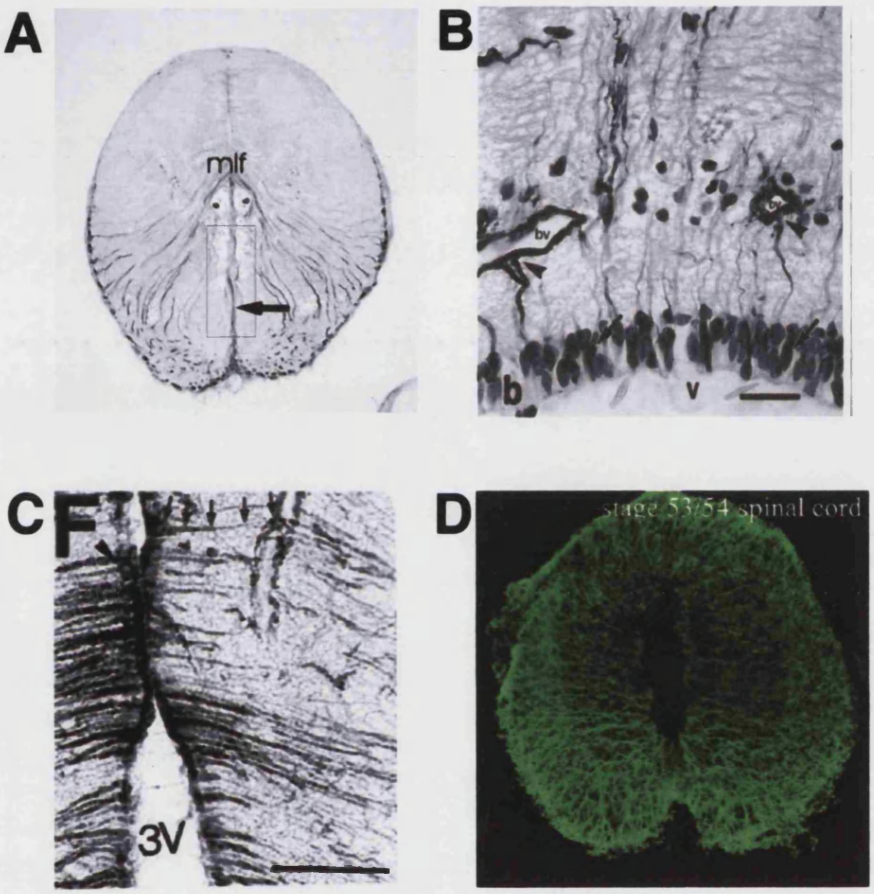
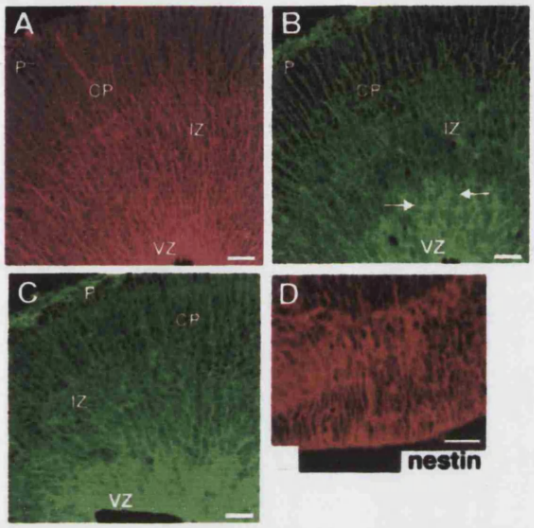

A
mlf
B
bv
b
v
C
3V
D
stage 53/54 spinal cord

Figure 1.5. Molecular markers of radial glia in rodents
Published markers of radial glia in the rodent telencephalon. All four markers show high expression in the cell bodies of the ventricular zone (VZ) and in basal radial glial fibres in the intermediate zone (IZ) and cortical plate (CP).
A. Radial Cell-2 (RC2) immunostain in a frontal section of mouse E16 cortex. Adapted from Hartfuss et al., 2001.
B. Brain lipid-binding protein (BLBP) staining in a frontal section of mouse E16 cortex. Adapted from Hartfuss et al., 2001.
C. Astrocytic glutamate transporter (GLAST) immunostain in a horizontal section of mouse E15 cortex. Adapted from Hartfuss et al., 2001.
D. Nestin immunostain in a section showing the boundary between cortex and lateral ganglionic eminence at E12.5. Adapted from Gaiano et al., 2000.
Scale bar: 50μm.


A
P
CP
IZ
VZ
B
P
CP
IZ
VZ
C
P
CP
IZ
VZ
D
nestin

E16 in the cortex at least, the majority of VZ cells express both RC2 and GLAST, with some of these cells also expressing BLBP (Hartfuss et al., 2001). The extracellular matrix molecule tenascin-C is strongly expressed in the cortical VZ (Bartsch et al., 1992 and Götz et al., 1998). Vimentin is a cytoskeletal protein that is expressed by a number of glial types including radial glia (Dahl et al., 1981, Bovolenta et al., 1984, Pixley and de Villis, 1984 and Kamei et al., 1998). Vimentin is also expressed by radial glia in birds, and unlike mammals, vimentin-positive radial glia can be detected in birds in adulthood (Alvarez-Buylla et al., 1987).

Radial glia can also be recognised in the CNS at the level of the electron microscope. In foetal mouse and human brain tissue, radial glia are largely electron-lucent, with abundant microfilaments and smooth ER, whilst their processes contain glycogen granules (Swarz and Oster-Granite, 1978, Choi and Lapham, 1978 and Choi, 1981). Radial glial somata also possess a short cilium that projects into the ventricle, although this feature is shared with neuroepithelial cells, ependymal cells and subventricular zone astrocytes (Tramontin et al., 2003).

Patterns of Division and Specification of Neural Progenitors in Vertebrates

Neuroblast-Glioblast Hypothesis

The cells of the CNS are divided into two main types, neurons and glia. It has been long believed that the two cell types are derived from a separate lineage, i.e., they are descendants of different pools of progenitors. This is the traditional view that was first postulated by histologists of the late Victorian era who did not have the benefit of live imaging; at the time it was not conceived possible that cells with such diverse appearance could be derived from the same cell, so it was believed that neurons and glia were generated from separate progenitor stocks. This was known as the 'germinal cell-spongioblast' theory of lineage, a model championed by, among others, Wilhelm His and Santiago Ramón y Cajal (see Jacobson, 1991 and references therein). In modern times this is usually referred to as the 'neuroblast-glioblast' model. The debate over cell specification and lineage is not new, since there have been views to the contrary since the model was first described; for example, Schaper long held that both neurons and neuroglia were derived from the same progenitor (Schaper, 1897). More recently, by analysis of tritiated thymidine incorporation, Setsuya Fujita postulated that in the cortex

the same progenitors gave rise to both neurons and glia by switching their potential from neuronal to glial (Fujita, 1963). He called this multipotential progenitor a 'matrix cell' and described that the embryonic VZ was made up entirely of these cells. Like Schaper long before him, Fujita's views appear to have been before his time, and his model was dismissed by many of his contemporaries (conclusions of the Boulder Committee, 1969) just as Schaper's ideas were by His and Ramón y Cajal. Instead, it is the neuroblast-glioblast hypothesis of diverged lineage that has dominated developmental neurobiology thinking, and it is only recently that this model is being reviewed due to new experimental evidence.

Evidence for Multipotentiality

Following the advent of retroviral-mediated gene transfer technology (Sanes et al., 1986 and Price et al., 1987) many studies have been devoted to clonal analysis of VZ progenitors. In 1987 Turner and Cepko demonstrated that single progenitors in the newborn rat retina could give rise to clones consisting of two different types of neuron and a Müller glial cell; in other words, in the retina at least, both neurons and glia can be derived from the same cell (Turner and Cepko, 1987). This was one of the first studies to show good evidence for neurons and glia derived of the same CNS progenitors. These data were both corroborated and expanded by clonal analysis using single-cell fluorescent dextran injection, where Wetts and Fraser showed in the frog retina, that single progenitors labelled in the optic vesicle can give rise to all the differentiated cell types in this tissue, with many clones containing five of the six different retinal cell types (Wetts and Fraser, 1988). On the other hand, some clonal analyses in the forebrain indicated that in the cortical VZ most progenitors were dedicated to producing either neurons or glia, but not both. Luskin and colleagues showed that almost all their clones were composed of one single cell type (Luskin et al., 1988). A later study by Luskin, additionally using EM to aid phenotyping, found that cortical clones were almost all composed of one of four cell types: pyramidal neurons, non-pyramidal neurons, oligodendrocytes and astrocytes (Luskin et al., 1993). However, other notable early studies suggested multipotentiality was actually a real phenomenon in the cortical VZ. In Price and Thurlow's clonal analysis of E16 cortical progenitors, some mixed clones contained neurons and putative oligodendrocytes ('horizontal cells') and one clone was

composed of neurons, horizontal cells and astrocytes (Price and Thurlow, 1988). These data were corroborated by a later study carried out by Williams and colleagues who detected, amongst a majority of single cell type clones, a minority of bipotential progenitors that gave rise to both neurons and oligodendrocytes, the so-called N-O lineage (Williams et al., 1991). In another study, although the authors concluded that each cell type was generated from a separate dedicated population of precursors, the data of Grove and colleagues describe 10% of their observed clones contained both neurons and glia, and of these, 4% with neurons, astrocytes and probable oligodendrocytes (Grove et al., 1993). Mione and colleagues reported that of clones observed to contain glia, a third contained both astrocytes and pyramidal neurons (Mione et al., 1997).

Other clonal analyses carried out in the chicken have predominantly supported the multipotential progenitor hypothesis. In the spinal cord, Leber and colleagues showed that although glia-only clones predominated, those that contained neurons were often mixed with astrocytes, oligodendrocytes or ependymal cells (Leber et al., 1990). The Leber study also showed that clones contained different types of neuron, including motorneurons, interneurons and preganglionic autonomic neurons; this contrasts with the findings of Parnavelas and colleagues who reported that in the mouse cortex, pyramidal and non-pyramidal neurons were almost always found in separate clones (Parnavelas et al., 1991). Studies in the chicken optic tectum (Galileo et al., 1990, Gray and Sanes, 1992) and the telencephalon (Szele and Cepko, 1996) produced mixed neuronal, oligodendrocyte and astrocyte clones. In the diencephalon, mixed astrocyte and neuronal clones were reported (Golden and Cepko, 1996). However, the conclusions that can be drawn from clonal analyses are restrained by several factors. Firstly, it is not possible to tell the exact lineage of each of the cells within the clone, if it contains three or more cells. Second, cells found close to one another are often assumed to be clonally related, but may be descended from two different cells in close proximity that were infected at the same time. Third, cell migration and clone dispersal may result in misinterpretation of which cells are siblings and which are clonally unrelated. Walsh and Cepko described a mode of progenitor dispersal in clonal analysis of rat cortex where a third of labelled clones were dispersed over a field 500μm in size six days after infection, and a small minority were found to be spread over 2mm (Walsh and Cepko, 1993). By using PCR to unequivocally determine clones, Reid and colleagues describe

how sibling neurons showed little tendency to remain clustered in radial units and often cells found lying within radial clusters were not clonally related (Reid et al., 1997). These dispersals were probably due to the non-radial or 'orthogonal' mode of migration described in the cortex by O'Rourke and colleagues (O'Rourke et al., 1992). Given the findings of Walsh and Cepko, and the Reid study, the data from retrovirus-mediated clonal analysis can always be subjected to some debate. On the other hand, single-cell labelling with a fluorescent tracer reveals both cell lineage and any potential clone migration or dispersal, since all fluorescent cells are the descendants of a single cell. This technique of labelling progenitors is less ambiguous than clonal analysis by retrovirus infection, and although it is not possible in any model system where the embryo is not readily accessible to label single cells, it has been used quite successfully in chicken (Fraser et al., 1990, Clarke and Lumsden, 1993 and Clarke et al., 1998) and in the zebrafish (Lyons et al., 2003). The Lyons study was also notable in that the authors followed fluorescent-labelled CNS progenitors over multiple rounds of division, in the intact brain (Lyons et al., 2003).

In recent years the evidence weighing against the neuroblast-glioblast separation of lineage is increasing, and that the most likely situation is of a single pool of multipotent progenitors, the cells of which can alter their behaviour to produce neurons or glia depending upon temporal or diffusible signals. Holt and colleagues describe that it is most likely local environment and positional information rather than lineage or temporal signals that determines retinal progenitor cell fate (Holt et al., 1988). However, the retina may be a special case, and not indicative of the entire CNS. Over time, single cultured cortical progenitors switch their proliferative behaviour to produce oligodendrocytes instead of neurons, mimicking the temporal change from neurogenesis to oligodendrogenesis that occurs in the brain *in vivo* (Qian et al., 2000). The dissociated cells were almost totally divorced from diffusible molecules and intercellular signalling since they were cultured singly, in serum-free minimal medium, on plastic. Despite this, the cells still displayed the same temporal switch from producing neurons to oligodendrocytes, implying that these cells possess some kind of intrinsic timer.

Modes of Progenitor Cell Division

Since many clonal analyses described the likely existence of multipotent progenitors, these cells will most likely have to undergo asymmetric cell division at some point in their life. It has been known for a number of years that the progenitor of the Drosophila nervous system, the neuroblast, undergoes asymmetric cell division to give rise to a neuroblast and a ganglion mother cell (GMC); the neuroblast is therefore self-renewed whilst the GMC is capable of dividing only once more, producing either two neurons or a neuron and a glial cell (reviewed by Jan and Jan, 1994). Many researchers have tried to demonstrate the process of asymmetric divisions in the neurogenesis of vertebrates. Some of the earliest indications that asymmetric cell divisions were taking place in vertebrate CNS were from retrovirus-mediated clonal analyses. Several studies noted that their clones were often divided into those composed of single neurons and others made up of multiple cells, and the both clone types were distributed approximately equally. Turner and Cepko reported that approximately half their newborn rat retinal clones were made up of single rod photoreceptors, the rest multicellular (Turner and Cepko, 1987). Reid and colleagues found in the ferret cortex, 52% of their clones were single neurons and the remainder similarly composed of clones containing more than one cell (Reid et al., 1997). Noctor and colleagues reported that after GFP-encoding retrovirus infection of the sensorimotor cortex, an equal distribution of clones composed of either single progenitors in the VZ (49.8%) or groups of cells in the mantle zone was observed (Noctor et al., 2001). Since replication-incompetent retroviruses only integrate into one daughter of the infected cell, and given the half-and-half observed frequency of their clones, the hypothesis is that an asymmetric cell division would give rise to the single neuron and a progenitor that would subsequently divide again several times to produce multiple-neuron clones. An interesting study by Mione and colleagues combined clonal analysis with BrdU incorporation, quantifying the strength of BrdU signal in the nuclei of the cells in each clone (Mione et al., 1997). The cells within each clone were described as derived from symmetric or asymmetric cell divisions depending on their position and the relative strength of BrdU signal in their nuclei; generally, cells in a clone occupying the same layer with equal BrdU levels were thought to arise from symmetric divisions, whilst those which possessed higher levels of BrdU than its relatives, and often arranged in a radial manner, were assumed to be derived from earlier asymmetric divisions. By these criteria the authors describe that a mixture of both

asymmetric and symmetric modes of division takes place during cortical neurogenesis, but neither mode predominates in the generation of cortical neurons. More recently Noctor and colleagues performed a clonal analysis of mouse cortical progenitors using a retrovirus encoding GFP, examining separate specimens at staggered timepoints 1 – 3 days after injection, to build an inferred or intellectual timelapse of cellular behaviour within each clone (Noctor et al., 2001). They found that one day after infection, their clones were largely composed of a single radial progenitor cell with its soma in the VZ, and one other cell in a more basal position, either in the intermediate zone (IZ) or the cortical plate (CP). With time the clones always tended to retain one VZ progenitor, and possess more cells in the IZ or CP; these cells tended to be multipolar and appeared to be migratory. By 72 hours after infection, the clones contained on average, one radial VZ progenitor and three or four multipolar cells. Also, the authors noted that clones were arranged in 'radial units', with multipolar cells in close proximity with the radial, basal process belonging to the VZ progenitor. VZ progenitors, but not their multipolar siblings, were found to express nestin, vimentin and incorporate BrdU; conversely multipolar cells expressed β-tubulin, whilst the radial cells did not. All these data are highly suggestive of single progenitor cells dividing asymmetrically and repeating this behaviour over, to give rise to the clones observed. The authors note that it is not possible to tell if the progenitor was dividing in a pure progenitor-neuron fashion successively or, was giving rise to an intermediate progenitor that divides once symmetrically to give rise to two neurons.

An interesting study by Takahashi and colleagues applied mathematical modelling to cortical neurogenesis by quantifying how many cells in a given region are in the cell cycle and how this proportion changes over time (Takahashi et al., 1996). Cells that leave the cell cycle i.e., terminally differentiate, termed quiescent or 'Q' fraction, and cells which are still in the cell cycle, i.e., progenitors, called progenitor or 'P' fraction, were quantified over the eleven cell cycles that take place between E11 and E17 of embryogenesis. The increase in the Q fraction with time is not linear; rather, from E12 to E14 Q was calculated as increasing from 0.11 to 0.36, and from E14 the Q fraction increases in size at a higher rate, rising from 0.36 at E14 to 0.67 at E15 and 0.79 a day after that. This implies that early in neurogenesis, divisions where both daughters are P, i.e., progenitor-pair symmetric divisions, predominate, whereas the rate of increase in Q,

or postmitotic neurons, is comparatively quite low. This represents an early multiplication in the numbers of progenitors in the VZ, as a prelude to more widespread neurogenesis. Later, as the production of neurons starts in earnest the Q fraction increases, but this rate is fairly low since asymmetric P-Q divisions predominate, simultaneously maintaining the progenitors of the VZ and making neurons. Towards the conclusion of neurogenesis, almost all remaining P cells become Q, and become terminally differentiated through Q-Q divisions, or symmetric, neuron-pair terminal cell divisions. Further modelling of neurogenesis by the same laboratory used the data of P and Q fraction numbers from the Takahashi study combined with retroviral analysis to show the proportion of cells that remained in the VZ out of the total cells in each clone (Takahashi et al., 1996 and Cai et al., 2002). The observed numbers from the retroviral experiment fitted closest the model they first described in 1996, with symmetric P-P, asymmetric P-Q and symmetric Q-Q divisions occurring throughout the entirety of neurogenesis, with the only variable being the change in the ratio of the three modes of division (Cai et al., 2002).

Mathematical modelling and circumstantial evidence from clonal analyses have indicated that asymmetric cell divisions do take place in vertebrate neurogenesis. However, using new brain slice culture techniques combined with timelapse microscopy, it is now possible to observe progenitor cell behaviour real-time in an environment closer to the in vivo situation. Miyata and colleagues cultured mouse cortical slices in which VZ progenitors had been labelled with DiI, and recorded timelapse movies of their cell divisions (Miyata et al., 2001). Firstly, the authors reported that a fine radial process in contact with the pial surface was maintained throughout M-phase. Additionally, out of 42 cell divisions they observed, in 36 cases this pial process was inherited by one daughter cell, which went on to migrate out of the VZ and into the IZ. The authors describe these mitoses as progenitor-neuron asymmetric divisions since one daughter generally remained at the ventricular surface and its basal process-inheriting sister cell was often (65%) found to lie vertically more than 20μm away from it. For further evidence of the neuronal phenotype of the distal daughter cells, eleven were subsequently stained for HuC protein, of which nine were immunopositive. However, other than its position in the VZ, progenitor phenotype was not confirmed in the other daughter cell, for example, the cells were not observed for

longer to see if they divided again. A limitation of the cortical slice culture system is that brain slices often degenerate after approximately three days in vitro (T. Miyata, personal communication) so cells cannot be followed over longer periods of time.

The evidence for asymmetric cell division in mammalian neurogenesis is increasing. However, the direct observation of mammalian neurogenesis is prevented by internal development: in order to watch cell divisions as they take place, mammalian CNS tissue must be placed in some form of ex vivo environment. As an alternative, the zebrafish is an excellent model system to observe vertebrate CNS progenitor cell divisions occurring in situ, in an intact brain. No other vertebrate model system offers the same rapid, external development combined with optical translucence that is ideal for timelapse imaging of neurogenesis. Lyons and colleagues recently used the zebrafish to characterise neurogenesis in vivo in the hindbrain (Lyons et al., 2003). Using single-cell lineage tracing, they showed that in the first two days of embryonic development only 11% of neurons are born from asymmetric, neuron-progenitor cell divisions, and that 84% of hindbrain neurons born during this period are derived from symmetric, neuron-pair terminal divisions. Therefore repeated asymmetric divisions generating the majority of neurons in the CNS may not be a feature common to all regions of the CNS, or to all species. This goes against many currently accepted theories of developmental neurobiology, where asymmetric neuron-progenitor cell divisions are considered to be the major neurogenic event in all vertebrates (see review by Wodarz and Huttner, 2003).

Radial Glial As Neural Progenitors

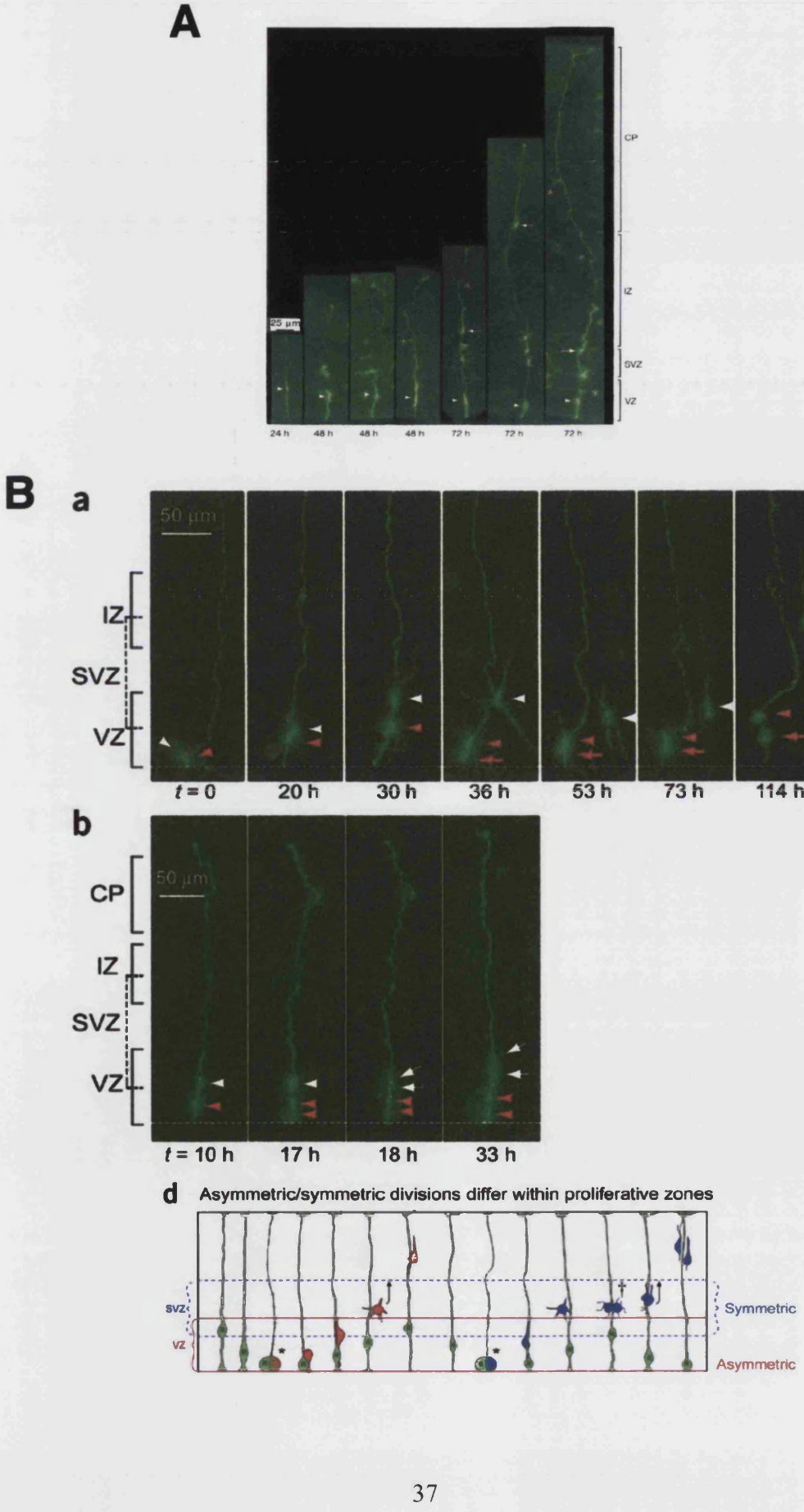
Early studies of radial glia in the developing brain centred on their patterning function, how these cells acted as a guiding rail for the migration of newborn neurons (Rakic, 1971a, 1971b and 1972 and Levitt and Rakic, 1980). Despite the subsequent demonstration of GFAP-positive mitotic figures at the ventricular surface in the macaque cortex (Levitt et al., 1981) it was conceptually not considered possible for radial glia to both divide and retain their neuronal guidance function (Schmechel and Rakic, 1979b). However, since the publication of a series of papers over the last five years, the traditional thinking behind radial glial biology has become altered quite radically.

Clonal Analyses and Timelapse Studies of Radial Glia

In February 2001, Noctor and colleagues published their clonal analysis of cortical progenitors (Noctor et al., 2001). Here the authors used a retrovirus encoding for GFP, and examined their animals 1 – 3 days after infection. They observed that after one day, clones were generally comprised of one progenitor cell in the VZ or one cell in the mantle layer, suggestive of asymmetric neuron-progenitor cell division. The progenitor cells were invariably bipolar and radial in morphology, possessing processes in contact with the ventricle and the pial surface, with their cell body in the VZ, the classical morphology of radial glia. After two days, two or three-cell clones appeared, and in such clones there was always one progenitor and one or two cells in the mantle zone, with their cell bodies in close attachment to the radial process of the progenitor (Figure 1.6). With longer timelapse the number of process-associated cell bodies increased but the single VZ progenitor remained. Immunostaining showed that GFP-expressing, radial VZ progenitors expressed the radial glial markers vimentin and nestin; conversely, multipolar mantle layer cells were Tuj1-positive. This was the first study to show, by clonal analysis, that radial glia were giving rise to neurons in the mouse cortex. Additionally, the single radial glia:multiple neurons ratio was highly indicative of repeated asymmetric cell divisions (see previous section).

In June of the same year Nobuaki Tamamaki and colleagues used an adenoviral approach to generate GFP-expressing clones of cortical VZ progenitors in mouse (Tamamaki et al., 2001). Animals were killed at sequential intervals over a period of four days – 10, 18, 48 and 96 hours after infection – and the different labelled cell types were counted. Eighteen hours after infection, radial glia comprised 50% of labelled cells, with 30% multipolar cells in the IZ or CP. Forty-eight hours post-infection, this proportion changed to 12.5% radial glia, 66% multipolar cells and 17% were differentiated neurons. Three days after infection, 96.5% were neurons and the remainder radial glia. GFP-expressing radial glia expressed RC2 and nestin, and incorporated BrdU. The authors also noted that many multipolar cells were observed in the subventricular zone (SVZ) at timepoints between 10 and 48 hours post-infection. They speculate that these multipolar cells were immature neurons 'pausing' in the SVZ, as if receiving positional information or cues to direct their correct axon extension or apicobasal position, before fully differentiating into mature neurons. Also, the authors

Figure 1.6. Clonal analysis and timelapse studies of radial progenitors in cortex
A. Adapted from Noctor et al, 2001. Mice were injected with a retrovirus encoding GFP at E16 and killed 1 – 3 days post-infection. This series is an inferred or intellectual timelapse since it is a composite of seven different specimens killed at the stage indicated. The number of GFP-expressing cells increases with the length of time the embryo is left to develop following retroviral infection. In every clone only one cell (arrowhead) is ever observed to show radial morphology with processes in contact with both ventricular (bottom of picture) and pial (top) surfaces. The authors hypothesise that this radial cell is generating the other cells through repeated rounds of asymmetric division.
B. Adapted from Noctor et al, 2004. Here the same group confirm their earlier hypothesis by recording timelapse movies of GFP-expressing progenitors in rat cortical slice culture. a) A single radial progenitor (red arrowhead) is observed to undergo cell division, giving rise to another progenitor and a neuron (white arrowhead) which migrates out of the VZ and into the IZ. The progenitor state of the radial cell is confirmed as it is observed to undergo a second cell division (red arrow and arrowhead). b) Cell divisions also take place in the SVZ. Here two cells in the same radial unit undergo cell division, one in the VZ (red) and the other in the SVZ (white). The authors hypothesise that the more basal cell at t=10h is an intermediate precursor born of an asymmetric cell division. This intermediate precursor then migrates to the SVZ where it undergoes another cell division (white arrows). The apical cell at t=10h goes on to divide in the VZ again, indicating that this may be a self-renewing progenitor. Taken together with other timelapse data and phenotyping the various cells by antibody staining, the authors describe a lineage similar to that of the Drosophila neuroblast (d).


A
CP
IZ
SVZ
VZ
25 µm
24 h
48 h
48 h
48 h
72 h
72 h
72 h
B
a
50 µm
IZ
SVZ
VZ
t = 0
20 h
30 h
36 h
53 h
73 h
114 h
b
50 µm
CP
IZ
SVZ
VZ
t = 10 h
17 h
18 h
33 h
d
Asymmetric/symmetric divisions differ within proliferative zones
SVZ
VZ
Symmetric
Asymmetric

compared previously published BrdU incorporation data (Takahashi et al., 1996) with the temporal proportion of GFP-expressing radial glia out of all GFP-expressing cells in their animals, and showed how their observed numbers fitted the theoretical Takahashi model closely (see Takahashi et al., 1996 and Cai et al., 2002).

In September 2001 Takaki Miyata and colleagues published an interesting study of radial glia in cortical slice culture (Miyata et al., 2001). Here the authors randomly labelled cortical progenitors with DiI, and recorded timelapse movies of their cell divisions. In addition to observing interesting asymmetries in cell division dynamics (see previous section) the authors show that their dividing progenitors express the radial glial marker RC2, corroborating the 'neurogenic radial glia' data of Noctor and Tamamaki. However, possibly the most interesting result of the study was the apparent inheritance of the basal radial glial fibre by the neuronal daughter in a radial glial asymmetric cell division, observed in 85% of recorded cases. The authors dubbed the newborn neuron a 'radial neuron' due to its radial morphology imparted by the inherited radial glial process. The authors hypothesise that the newborn neuron uses this radial process as a guide during its inside-out radial ascent in the mantle layer.

In 2002 Noctor and colleagues published another study of neurogenic cortical radial glia (Noctor et al., 2002). By following the same infection protocol as their earlier study, but allowing the animals to develop to P6, they showed how the majority of radial-glia derived neurons were pyramidal neurons inhabiting the upper layers of the cortex. The authors also visualised a transitional form of GFP-expressing cell that appeared to be radial glia in the process of transforming into astrocytes. Here, they presented good evidence for their challenging hypothesis that most, if not all, VZ progenitors in the embryonic mouse cortex were in fact radial glia. They took recordings from 102 randomly selected VZ cells, and found that all of them possessed the intracellular electrophysiological characteristics of progenitors. Each of these cells was also filled with Lucifer Yellow dye to reveal their cell morphology, and all were found to be bipolar, have a VZ-residing soma and extend their basal process to reach the pial surface, except in one instance, where the process terminated in the cortical plate. To show what proportion of proliferating cells radial glia contributed in the VZ, cortical sections prepared from animals pulsed with BrdU at E12, E15 and E18 were stained with the radial glial marker vimentin. Vimentin-expressing cells accounted for no less

than 98% of BrdU-incorporating S-phase cells at each timepoint. Thus the data from this study, in conjunction with the results from their previous paper from 2001, strongly indicate that the great majority of VZ cells are in fact radial glia, or at least radial progenitor cells with processes in contact with both ventricular pial surfaces.

Early this year Noctor and colleagues published the latest in their series of 'neurogenic radial glia' papers (Noctor et al., 2004). Using timelapse imaging of GFP-expressing single radial glia in rat cortical slices, they describe various migratory aspects of cortical radial glial-derived neurons (Figure 1.6). In particular, Noctor and colleagues describe 'four phases of migration' of radial glial-derived, newborn neurons. Following their birth in the VZ, many (65%) neurons migrated radially into the SVZ, underwent a temporary arrest, migrated in a retrograde fashion toward the ventricle and often back into the VZ, before concluding with their final radial migration into the cortical plate. Additionally, Noctor and colleagues were able to reconstruct the lineage trees of some of their dividing radial glia, a feat they were unable to do, but speculated on, in their 2001 study. When dividing, radial glia were shown to give rise to one radial glia, thereby self-propagating, and often the other daughter was an intermediate progenitor which went on to divide once more in a terminal symmetric manner within the SVZ, to make two neurons. Amazingly, this lineage is almost identical to that of *Drosophila* neuroblast and its transient daughter, the ganglion mother cell, the only difference being the terminal division of the *Drosophila* GMC can sometimes be an asymmetric neuron-glia division (Jan and Jan, 1994), whilst those observed to take place in the SVZ by Noctor and colleagues were almost all symmetric neuron-neuron in nature. Whether or not the same mechanisms as in *Drosophila* are dictating this stereotyped lineage has yet to be tested, but it is certainly a quite interesting development.

Transgenic Methods to Fatemap Radial Glia In Vivo

Malatesta and colleagues used a different approach to demonstrate that radial glia are neurogenic *in vivo* (Malatesta et al., 2003). This was the first study to look at the ultimate fate of cortical radial glia as a population in the mouse brain, phenotyping their progeny at postnatal stages using antibody markers. Earlier *in vitro* studies in the same laboratory had shown that radial glia isolated from the cortex could give rise to neuronal progeny: two thirds of the descendants of isolated E16 radial glia expressed β-tubulin

(Malatesta et al., 2000). Here the authors employed a Cre-recombinase transgenic method to label all the progeny of radial glial cells with LacZ. Previous studies had shown that 10kb of promoter sequence from cloned human GFAP could drive GFP expression in astrocytes in adult mice (Zhuo et al., 1997 and Nolte et al., 2001) and in the same transgenic, it was found that radial glia also express GFP in the embryonic brain (Malatesta et al, 2000). Malatesta and colleagues crossed the hGFAP-Cre line (Zhuo et al., 2001) with the ROSA26R indicator strain, to provide a total fatemap of all radial glia in the CNS. In hGFAP-Cre;R26R mice the authors found that at P21, many neurons in the grey matter of the cortex were β-galactosidase positive, i.e., derived from transgene-expressing radial glia. In the cortex, approximately 86% of all radial glial progeny were neurons, 80% of which were identified by the authors as pyramidal projection neurons by their glutamate expression. The remaining 6% was made of interneurons, as identified by parvalbumin, calretinin or calbindin expression. Almost all the other progeny (14%) was comprised of glia, identified by CC1, AN2 or BLBP expression. In the ventral forebrain, a different story was evident. A far smaller proportion of β-galactosidase-positive cells in the striatum were neurons (28%), comprised of 10% projection neurons and 18% interneurons. The remainder were glia, with CC1-expressing oligodendroglia making up the majority (60%). Therefore a great difference in the fate of radial glial progeny was observed between dorsal and ventral telencephalon. The authors go on to demonstrate that the differences in fate between radial glia of the cortex and the ganglionic eminences were cell autonomous by using an *in vitro* assay. GFP-expressing radial glia from the cortex or ganglionic eminence (GE) were isolated by FACS from the hGFAP-GFP transgenic mouse and plated onto a layer of feeder cells isolated from rat cortex or GE. Cortical radial glia produced an almost equal proportion of neurons to glia, whilst GE-derived radial glia gave rise to almost entirely non-neuronal progeny. These ratios were maintained in both homotypic (i.e., cortical radial glia plated onto a cortical feeder layer or GE radial glia on GE feeder cells) and mixed (cortical radial glia on GE feeders, or *vice versa*) co-cultures. One curiosity of the Malatesta hGFAP-Cre;R26R fate map is Cre-positive GFAP-positive astrocytes: practically none was observed. Since differentiation into stellate astrocytes is a well-described perinatal fate for mammalian radial glia (Choi and Lapham, 1978, Schmechel and Rakic, 1979a, Choi, 1981, Voigt, 1989, Gaiano et al., 2000 and Schmid

et al., 2003) then the quantification of radial glial progeny in the Malatesta study is skewed by the absence of these cells; the most common glial fate recorded was CC1-expressing oligodendrocytes.

Recently Anthony and colleagues recently published a report that used a similar methodology to that of Malatesta and colleagues (Anthony et al., 2004). The Heintz laboratory had previously shown that 1.6kb of promoter sequence from the gene encoding brain lipid-binding protein (BLBP) drove GFP expression in radial glia and astrocytes (Feng and Heintz, 1995). Anthony and colleagues showed that expression of GFP in the BLBP-GFP transgenic mouse recapitulated the endogenous expression of BLBP. They then used a BLBP-Cre transgenic mouse, crossed with the ROSA26R indicator line, to fate map all BLBP-expressing radial glia. The results were interesting, since β-galactosidase is expressed in the forebrain as early as E10.5, whereas the reporter of Malatesta study's transgene is only detectable from E14. Analysing BLBP-Cre;R26R mice at P45, the authors found β-galactosidase-positive neurons in all regions of the brain examined. In the cortex, 98.7% of NeuN-positive neurons were β-galactosidase-positive; in the basal ganglia, 71.4% and the thalamus 98.1%. Therefore unlike the report of Malatesta and colleagues, the great majority of neurons in the ventral telencephalon was also found to be derived from radial glia. Another result in apposition to the Malatesta study was the much greater number of interneurons in the cortex – presumably originating from the ganglionic eminences – that were also radial glia-derived: a great proportion of interneurons expressing calbindin, calretinin or parvalbumin were found to be β-galactosidase-positive (87.2%, 80.7% and 92.5% respectively). The authors do however report that radial glia-derived stellate astrocytes did not report in their transgenic system, just as occurred in the study of Malatesta and colleagues; this is therefore probably a technical consideration of the Cre-recombinase system employed by both studies. However, this does not detract from the observations of neurogenesis made in this paper. Using BLBP as a marker of radial glia appears to have covered more of the early neurogenic period of the forebrain than the hGFAP-Cre transgenic. Neurogenic radial glia in the ventral telencephalon, the authors explain, were missed by the Malatesta study since neurogenesis in this brain area occurs largely before their transgene reports. By the time cortical radial glia would have begun to produce LacZ under the control of the hGFAP promoter at approximately E14, the radial

glia of the ventral forebrain should have already concluded their neurogenic period and instead have switched toward mostly gliogenesis. Because their transgene catches radial glia very early in development, Anthony and colleagues speculate that BLBP may be the earliest marker that discriminates differentiated radial glia from their neuroepithelial neighbours in the early VZ.

Radial Glia in Non-Mammalian Vertebrates

Although it is only recently that the neurogenic role of mammalian radial glia has come to light, evidence from non-mammals has suggested this for many years previously. More than twenty years ago Stevenson and Yoon reported radial glial mitosis in the CNS of adult goldfish (Stevenson and Yoon, 1981). Acute treatment with tritiated thymidine revealed label-incorporating cells in the ventricular zone of the lizard cortex, and when treated animals were allowed to recover for one month, labelled nuclei were found in the granule and inner plexiform layer (Lopez-Garcia et al., 1988). Alvarez-Buylla and colleagues observed that in canaries, radial glia reside in the same brain regions as 'hot spots' of adult neurogenesis takes place (Alvarez-Buylla et al., 1990). Observations of apparent radial glial-mediated neurogenesis were facilitated in non-mammalian vertebrates because these vertebrates generally undergo neurogenesis throughout adulthood. However, it is only with the recent mammalian radial glial research that the field has come to gather such great interest as it does now. The wide multipotentiality of radial glia as demonstrated by the fatemapping studies of Malatesta and colleagues and Anthony and colleagues (Malatesta et al., 2003 and Anthony et al., 2004) showed that this cell type is far more interesting than we first thought. Another intriguing facet of this field is the link between glia, adult neurogenesis and CNS regeneration.

Radial Glia, Astrocytes and Neural Stem Cells

Glia as Neural Stem Cells

The demonstration of neurogenic radial glia in mammals proved quite a development, yet in recent times there is an increasing amount of research that is challenging our views of many cells of the traditional glial lineage. The word glia is derived from the ancient Greek for 'glue'; these cells were considered to be a packing or supportive cell

that gave structure to the brain. Research in more modern times demonstrated that glia provided trophic and metabolic support to neuronal cells (for a summary of these traditional functions of glia, see Jacobson, 1991). Who would have thought that, but ten years ago, not only glia gave rise to neurons during embryonic development, but could also hold the key to CNS regeneration? In fact, as early as 1992 the possibility of radial glia acting in a stem cell-like fashion was raised in a clonal analysis in chicken optic tectum (Gray and Sanes, 1992). This was the first such retrovirus-mediated analysis to show clones containing radial glia. In this study, on examination of the frequency of single radial glia within these clones, and the temporal distribution of single radial glia, the authors hypothesised that it was the radial glia in their clones that were giving rise to neurons, astrocytes and other radial glia, and speculated about the stem cell-like nature of radial glia. However, it is only with recent studies in mammals that the link between radial glia and stem cells is coming to the fore. Radial glia have been shown to make neurons and oligodendrocytes (Malatesta et al., 2003 and Anthony et al., 2004) and astrocytes (Gaiano et al., 2000, and Schmid et al., 2003) in the embryonic rodent brain. They also divide by self-propagating, asymmetric cell divisions (Miyata et al., 2001, Noctor et al., 2001 and 2004). Therefore it could be tempting to describe radial glia as a neural stem cell. However, by my own definition of stem cells, mammalian radial glia do not meet the third criterion, which is immortality.

There are also several other types of glia that show some characteristics of stem cells, such as Müller glia and SVZ astrocytes. Fischer and Reh reported that following mechanical injury to the chicken retina, glutamate synthase-expressing Müller glia were observed to rapidly proliferate, and subsequently many of these cells were found to then express markers of retinal progenitors such as Pax6 or CASH-1, indicating some manner of de-differentiation event had taken place within these cells (Fischer and Reh, 2001). Label-retaining experiments showed that a small minority of these cells went on to later differentiate into neurons or Müller glia, but the majority remained in this progenitor-like state (Fischer and Reh, 2003). Specific ablation of ganglion cells using colchicine resulted in many Müller glia-derived differentiated neurons to express markers of ganglion cells and develop ganglion cell morphology, implying Müller glia were capable of specific or dedicated neuronal regeneration (Fischer and Reh, 2002).

In the adult mouse forebrain, an interesting stem cell story is unravelling. Retrovirus-mediated overexpression of Hes1 in cortical VZ was found to direct progenitors to an ependymal cell fate (Ishibashi et al., 1994). This finding did not appear to be of much interest until data from a group working at the Karolinska Institute in Stockholm were published in January 1999 (Johansson et al., 1999). Johansson and colleagues published a paper where they report stem cell behaviour of glia in the forebrain and spinal cord of adult rat. The authors labelled ependymal cells by intraventricular injection of DiI and ten days later, found that some fluorescent cells had apparently delaminated from the ependyma, appeared to be joining the rostral migratory stream and ultimately residing in the olfactory bulb. Following an incision injury to the dorsal spinal cord, cells in the ependymal layer were seen to undergo an increase in proliferation and 2 – 4 weeks later fluorescent cells were found to have spread throughout much of the spinal cord. Immunostaining showed that some of these cells were GFAP-positive astrocytes. However, this study attracted quite some criticism following its publication, including several direct refutations. Several months later Doetsch and colleagues responded with a paper that described subventricular zone (SVZ) astrocytes, and not ependymal cells, as the real stem cell in the adult mouse forebrain (Doetsch et al., 1999). They provided several lines of evidence for this: firstly, after treating animals with an antimitotic toxin 'Ara-C', which apparently selectively kills all newborn neurons and proliferating intermediate progenitors in the SVZ, it is SVZ astrocytes and not ependymal cells that upregulate their proliferation to replenish these lost cells. Second, using mCD24 as a marker of ependymal cells, long-term label-retaining experiments showed that ependymal cells did not retain BrdU over 2 weeks, and instead label was detected in cells of the SVZ. Third, using a GFP-transgene specifically targeted to GFAP-expressing SVZ astrocytes, it was shown that these cells contribute neurons to the olfactory bulb. More recent work from another laboratories have generally corroborated the Doetsch model and dismissed that of Johansson and colleagues. Laywell and colleagues cultured isolated ependymal cells and SVZ astrocytes, and found that whilst the former were unipotent, the latter could give rise to neuronal and glial cells *in vitro* (Laywell et al., 2000). Capela and Temple describe a secreted carbohydrate called Lewis-X (Lex) that is expressed by GFAP-expressing cells in the SVZ i.e., SVZ astrocytes, but is not found in the ependymal layer (Capela and Temple, 2002). LeX-

expressing cells were found to make neurospheres *in vitro*, whilst isolated ependymal cells did not. One possibility that may explain the discrepancy between the results of the Johansson study and that of Doetsch was the in the former, the authors may have inadvertently labelled SVZ astrocytes whilst labelling the ependymal layer with DiI. Although at the level of the light microscope the ependyma appears to be a complete barrier between the ventricular fluid and SVZ, EM has shown that SVZ astrocytes are in very close contact with the ependymal cells, with large areas of cell membrane in intimate contact between the two cell types (Doetsch et al., 1997) and some SVZ astrocytes have been observed to possess a small cilium that projects through the ependymal layer to the ventricular surface (Tramontin et al., 2003).

Notch Signalling and Glia

In the developing vertebrate CNS, Notch signalling has been studied the most for its role in maintaining cells in a progenitor state during neurogenesis (see Lewis, 1998). However, recent studies are beginning to reveal that Notch signalling also plays an important role in the specification of glia. *In vitro* experiments showed that overexpression of activated Notch1 (often referred to as ActN, or $Notch_{ICD}$) may promote the number of proliferating cells in the newborn rat retina, and these cells possessed Müller glial morphology, and expressed Müller glia markers (Bao and Cepko, 1997 and Furukawa et al., 2000). The Furukawa study also showed that overexpression of a dominant-negative form of Hes1, a downstream effector in the Notch signalling pathway, produced the opposite effect. In the zebrafish retina, an interesting study by Scheer and colleagues revealed how expression of activated Notch directed cells toward either a zrf1-expressing Müller glial phenotype or that of apparently undifferentiated cells (Scheer et al., 2001). Ganglion, amacrine cell and photoreceptor fate were greatly reduced, and sometimes even completely abolished. Previous studies in the retina have shown that activated Notch expression leads to an undifferentiated progenitor phenotype in the chicken and frog (Henrique et al., 1997 and Dorsky et al., 1995) or differentiation into Müller glia in the rat (Furukawa et al, 2000); this study showed that cells were directed to a mixture of the two fates. These different data garnered from different systems suggest that one of two things is happening. Either Müller glia can behave as stem cell-like retinal progenitors, de-differentiating in response to their circumstances

such as local injury, as the experiments of Fischer and Reh suggest (Fischer and Reh, 2001, 2002 and 2003) or that in the retina, there is a co-resident neuroepithelial-like stem cell that is so far, to us at least, outwardly indistinguishable from Müller glia.

Using the technique of ultrasound-guided *in utero* injection, Gaiano and colleagues showed that activated Notch expressed in the mouse telencephalon promoted the expression of radial glial markers RC2, BLBP and nestin in cortex and lateral ganglionic eminence (Gaiano et al., 2000). Recent studies have shown that radial glial cells expressing these markers in the forebrain are multipotential progenitors *in vitro* (Malatesta et al., 2000) and *in vivo* (Malatesta et al., 2003 and Anthony et al., 2004). However, another study showed that not only did activated Notch greatly reduce neuronal number and increased the number of astrocytes, but activated Notch expression also decreases the total cell number in many regions of the forebrain (Chambers et al., 2001). The authors speculate that in addition to promoting glial fate and inhibiting neuronal differentiation, Notch was retaining cells in a non-proliferative, quiescent stem cell-like state. This was especially marked in the olfactory bulb, where it was shown that almost no precursor cells migrated there from the anterior cortical SVZ, and the total cell number in the olfactory bulb was reduced forty times when quantified at P21.

Given our previous knowledge of Notch signalling (see Lewis, 1998) some researchers may be surprised to read of the role this pathway in the glial lineages. The traditional view of Notch in verterbate neurogenesis is that of restraining differentiation or retaining cells in a progenitor state. However, with our recently acquired knowledge of the progenitor-like characteristics that many glia possess, this concept becomes slightly less counter-intuitive.

Radial Glia as Neural Stem Cells: Experiments in Non-Mammals

There are some arguments that indeed the mammalian neural stem cell is the radial glia, although by my three criteria as defined at the beginning of this chapter, mammalian radial glia do not qualify, as they are not immortal. However, one could argue that some radial glia give rise to SVZ astrocytes, a certain subpopulation of which can act as stem cells in the adult forebrain of mice (Malatesta et al., 2003 and Doetsch et al., 1999). In general though, postnatal neurogenesis is very limited in mammals. For example, even if there are neural stem cells residing in the adult human CNS, they clearly do not

regenerate lost neurons, as sufferers of spinal cord injury, stroke or Parkinson's Disease will attest. In order to better study the mechanisms by which radial glia can act as stem cells in the adult brain some researchers have turned to the non-mammalian vertebrates for clues. Unlike mammals, it has been shown that amphibians, reptiles, birds and both classes of fish retain radial glia post natum (Stensaas and Stensaas, 1968a and 1968b, Zamora, 1978, Ahboucha et al., 2003, Alvarez-Buylla et al., 1990, Kálmán, 1997, 1998 and 2001, Kálmán and Gould, 2001) and research in the last the last two decades has demonstrated that teleost fish (Zupanc and Zupanc, 1992 and Zupanc, 1999), songbirds and non-songbirds (Alvarez-Buylla et al., 1987 and 1994 and Ling et al., 1997), reptiles (Lopez-Garcia et al., 1990) and amphibians (Chetverukhin and Polenov, 1993) all undergo neurogenesis in various regions of the brain during adult life. In teleost fish many of the newborn neurons in the adult are culled by apoptosis (Zupanc, 1999) and in songbirds the rate of adult neurogenesis differs seasonally and is subject to turnover (Nottebohm et al., 1994 and Alvarez-Buylla and Kirn, 1997) implying that this adult neurogenesis, much like that which takes place in embryonic development, is under tight control and performs specific functions. Also the capacity for adult neurogenesis has been linked with an additional ability for regeneration of the adult CNS following injury in fishes (Zupanc, 1999 and Zupanc and Clint, 2003), amphibians (Chetverukhin and Polenov, 1993) and reptiles (Wang and Halpern, 1982a and 1982b and Lopez-Garcia et al., 1992). However, possibly the most striking CNS regeneration reported in the literature to date is that of the neotenic amphibian Ambystoma mexicana, the axolotl, which requires special mention. It has been known for many years that the axolotl can completely regenerate its tail following amputation (see Holder and Clarke, 1988 and Chernoff, 1996). Within the regenerating tail, the spinal cord is completely restored to its pre-amputation state with time (Clarke et al., 1988). However, it was the recent study of Echeverri and Tanaka that demonstrated some remarkable regenerative behaviours of radial glia following tail amputation (Echeverri and Tanaka, 2002). The authors used a DNA construct encoding GFP under the control of 10kb of the human GFAP promoter (Nolte et al., 2001; see Malatesta et al., 2003) and electroporated this into perilumenal cells of the spinal cord. This method labelled between one and four radial glia in the regenerating spinal cord. Examining the tail at regular time intervals, the authors noted that some GFP-expressing radial glial cells were proliferating and their progeny

migrating toward the wound site. Later, some of these progeny were found to have exited the spinal cord and differentiated into muscle or cartilage. Other radial glia appeared not to undergo this event, and instead proliferated to produce more radial glia. hGFAP-GFP-expressing radial glia were found to be able to give rise to muscle, cartilage, neurons, neural crest, melanocytes and glia. This was the first study to demonstrate radial glia in the adult spinal cord behaving as pluripotent stem cells capable of producing not only cells of ectodermal origin to restore the spinal cord but also mesoderm-derived cell types to replace other structures in the tail. This property of radial glia has been to date limited to amphibian model systems, yet the potential rewards for being able to understand the molecular basis of such striking spinal cord regeneration, and being able to recapitulate it in higher vertebrates, are very great indeed.

Mechanisms of Asymmetric Division

Studies in Flies

In *Drosophila*, the crucial event regarding what fate a cell adopts in the developing nervous system is the asymmetric inheritance of intracellular determinants during cell division (reviewed by Doe and Technau, 1993, Jan and Jan, 1994 and Lu et al., 2000). When a cell undergoes mitosis, some proteins in the cytosol are divided unequally between the two daughter cells as the cytoplasm is split and shared between the two daughter cells. When a neuroblast, the neural progenitor of the *Drosophila* nervous system, divides, one daughter cell remains a neuroblast and the other daughter is a restricted precursor known as a ganglion mother cell (GMC). The GMC daughter goes on to divide only once more, producing either a pair of neurons or a pair consisting of one neuron and one glia. In addition to differences in size and position – the GMC is smaller, and migrates to a more basal position – there is also a difference in gene expression profiles between the two cell types. The neuroblast shows asymmetric protein localisation during cell division: in metaphase Bazooka and Inscuteable are localised to the apical half of the cell, whilst Miranda, Numb, Partner of Numb and Prospero localise to the basal cortex (see Jan and Jan, 1994, Knoblich et al., 1995 and Lu et al., 2000), and are inherited by the daughter cell that develops into a GMC. Of these, the proteins important in specifying the GMC are Prospero and Numb, whilst Miranda

and Partner of Numb are adapter molecules that help partition these proteins and establish polarity within the dividing NB (see Lu et al., 2000). This system of asymmetric inheritance of intracellular determinants mediates the generation of diverse progeny in the dividing neuroblasts of the *Drosophila* nervous system.

Evidence For Conservation in Vertebrates: Asymmetric Protein Distribution

Since asymmetric inheritance of intracellular determinants has been shown to be crucial in fate decisions of the progeny of *Drosophila* neuroblasts, many researchers have sought whether or not similar mechanisms are conserved in the mammalian brain. In the progenitors at the ventricular surface of the embryonic cortex, several studies have shown that junctional complexes in the plasma membrane ring the apicalmost portion of the cell (Huttner and Brand, 1997, and references therein). These complexes can recruit or partition cytoplasmic proteins such as PAR-3, a mammalian homologue of *Drosophila* Bazooka, to the apical half of the progenitor cell (Manabe et al., 2002). Notch and Numb are two other proteins that have been shown to be involved in asymmetric division, and are conserved in *Drosophila*, *C elegans* and many vertebrates (see Lu et al., 2000 and references therein). Chenn and McConnell demonstrated that Notch1 was localised to the basal side of progenitor cells in the cortical VZ of ferret (Chenn and McConnell, 1995). In 1996, two laboratories independently cloned a mouse homologue of *Drosophila* Numb (Zhong et al., 1996 and Verdi et al., 1996) and ever since, an amusing and intriguing field-within-a-field has developed (reviewed by Zhong, 2003). Zhong and colleagues visualised Numb protein with an antibody and reported that it was restricted to the apical domain of progenitors in the cortex, and that a cell that inherited Numb from a perpendicular cell division would remain a progenitor. However, Verdi and colleagues were unable to detect this localisation, instead finding it distributed evenly in the cytosol; this may have been due to the antibody used in their study cross-reacting with a similar protein called Numblike (Zhong et al., 1997; personal communication, W. Zhong). Later, another group cloned Numb in the chicken and demonstrated that not only was the protein apparently localised to the basal half of dividing progenitors, it was shown to be able to interact with the cleaved intracellular domain of Notch (Wakamatsu et al., 1999). This was an attractive result since it would suggest the cell destined to become the neuron in an asymmetric cell division would

asymmetrically inherit Numb protein, and that this Numb protein would inhibit Notch signalling and permit differentiation to take place. In the retina, different workers are also unable to agree upon the distribution of Numb protein: in the rat, Cayouette and colleagues visualised Numb to the apical surface of dividing progenitors whilst in the chicken, Silva and colleagues managed to find it localised basally (Cayouette et al., 2001 and Silva et al., 2002). The mouse Numb knockout has also proved enigmatic, since yet again, different labs have produced conflicting data. Homozygous mutants in Numb raised by Zhong and colleagues displayed a very mild phenotype, with outwardly few defects in neurogenesis (Zhong et al., 2000). The knockout mice created by Zilian and colleagues showed *spina bifida*, loss of DRG sensory neurons and spinal cord motorneurons and invariably died by E11.5 (Zilian et al., 2001). However, the double Numblike:conditional-Numb knockout mouse possesses a neurogenic phenotype (Petersen et al., 2002) which would support the notion that Numb is a determinant of progenitor, rather than neuronal fate, as initially proposed in 1996 by Zhong and colleagues (Zhong et al., 1996).

Evidence For Conservation in Vertebrates: Cell Biology Experiments

The study of Chenn and McConnell was one of the first to correlate cleavage plane with cell fate in progenitor cell divisions in cortical slice culture (Chenn and McConnell, 1995). Here, the authors labelled progenitors with DiI and observed their cell division behaviour. In the same study immunostaining showed that Notch1 was localised to the basalmost extreme of dividing cells at the cortical ventricular zone, which they hypothesised was a determinant of neuronal fate. Chenn and McConnell reported that cell fate was being determined by cleavage orientation: a cell division in the same plane as the ventricular surface gave rise to two progenitors through a symmetric inheritance of Notch1 protein. Conversely, cell divisions that were perpendicular to the plane of the ventricle were reported to give rise to a progenitor and a neuron. The basal daughter would inherit the basally-concentrated Notch protein and go on to develop into a neuron. The seductive simplicity of the system won the Chenn and McConnell model many supporters, but some other researchers received the authors' conclusions with less enthusiasm. Detractors of the study would point out that no antibody staining of either

putative progenitors or neurons was carried out to confirm their phenotype; putative progenitor cells were not followed to see if they underwent another cell division.

Since the publication of the Chenn and McConnell study, other groups in other systems have tested their hypothesis. Timelapse videos of the *in vitro* culture of newborn rat retina by Cayouette and Raff appear to corroborate the McConnell model (Cayouette and Raff, 2003). The authors devised an elaborate system of bending the explanted retina in half in order to image GFP-expressing single progenitors through the folded tissue. The authors analysed the orientation of 49 progenitor cell divisions, in 58% of which the plane of division was parallel to plane of the ventricular surface and 34% where the division was perpendicular. The progeny of horizontal cell divisions apparently developed into photoreceptors, and those of perpendicular divisions differentiated into cells of differing types; however, the authors phenotyped the daughter cells by nuclear size only and did not perform any molecular or marker characterisation.

The zebrafish is proving to be an excellent model organism for *in vivo* timelapse imaging. Recently Das and colleagues recorded timelapse movies of dividing progenitors in the retina of zebrafish (Das et al., 2003). Here the authors report that all observed cell divisions were in the plane of the ventricle, and no perpendicular mitoses were ever observed. Lyons and colleagues also used the zebrafish to analyse division orientation, this time in the hindbrain (Lyons et al., 2003). Here, the authors showed that in cell divisions that generated a neuron and a progenitor, the cleavage of the daughter cells occurred within the plane of the ventricle i.e., not apicobasally, in 98% of observed cases. However, even if one does not include the disputed Chenn and McConnell hypothesis, there is clear evidence that in vertebrates a) proteins can be distributed within the VZ progenitor cell asymmetrically, such as to an apicalmost domain maintained by junctional complexes and b) many of these proteins have been shown to be important in asymmetric cell divisions in invertebrates. If all zebrafish hindbrain progenitor cell divisions are of the horizontal nature, how can these proteins such as PAR-3 or Numb be distributed asymmetrically during cell division? Huttner and Brand describe a convincing case for asymmetric cell divisions that does not require the cell cleavage to occur parallel to the plane of the ventricle (Huttner and Brand, 1997). With many proteins localised to the apicalmost extreme of cells at the ventricular surface, even a very small deflection in cleavage plane, from vertical to oblique, would

result in unequal sharing of the apical domain between the two daughter cells (Figure 1.7). The angle of cleavage may be deviated from the vertical by only a few tens of degrees, so that it would be undetectable by the imaging procedures used by Lyons and colleagues. Perhaps in the coming years, with the advent of microscope technology with even greater resolution and performance, it will be possible to determine angle of division cleavage in dividing progenitors and test the Huttner and Brand hypothesis.

Radial Glia and Neuronal Migration

Early Models

Radial migration, or the migration of newborn neurons from ventricular or germinal zone to mantle layer is one of the most fundamental processes of CNS patterning. In the cortex, it has been shown that the reelin signalling pathway is crucial for the correct 'inside-out' layering of neurons in this brain region; mutations in this pathway lead to severe neurological defects (see Rice and Curran, 2001). Golgi staining of cortical preparations revealed apparently binucleate cells with processes in contact with both ventricular and pial surfaces; the authors hypothesised that these cell bodies were 'sliding' in some way along these processes to reach the cortical plate (Berry and Rogers, 1965). Later, Pasko Rakic's definitive studies of radial glia in *Macacus* describe this cell type as a scaffold to guide newborn neurons migrating to their appropriate apicobasal target (Rakic, 1971a, 1971b and 1972; for review see Rakic, 1990 and 2003). Rakic also demonstrated that radial glial processes in his model system expressed GFAP (Levitt and Rakic, 1980). Rakic's studies utilised painstaking computer reconstruction of serial sections, imaged by electron microscopy, to describe the intimate membrane contact between newborn neurons and radial glial processes in both cerebellum and neocortex of foetal macaque (1971a and 1971b). However, since radial migration is a dynamic movement of living cells, other workers have attempted to visualise the process of radial migration actually taking place. The laboratory of Mary Hatten developed an *in vitro* primary culture system of dissociated cerebellar cells to model the migration of granule cells along Bergmann glia processes, and record timelapse movies of this movement (Edmondson and Hatten, 1987, Gasser and Hatten 1990a and b). Edmondson and Hatten found that migrating neurons extended a thick leading process along the glial fibre which rapidly extends and retracts (Edmondson and

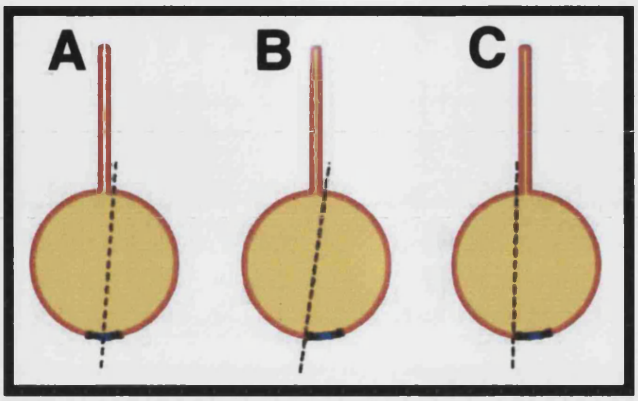


Figure 1.7. Membrane polarity, cleavage plane and division symmetry
Hypothetical models of cleavage plane orientation during neural progenitor cell division, adapted from Wodarz and Huttner, 2003. Vertical or near-vertical cleavages can result in asymmetric inheritance of intrinsic cell fate determinants. Red, cell membrane, black, junctional complexes and blue, apical cell membrane domain containing putative cell determinant proteins.
A. Near-vertical cleavage plane bisecting the apical cell membrane evenly, resulting in an equal distribution of proteins in this domain.
B. Cleavage plane deflected to include the apical membrane domain within one daughter cell only. The basal process is inherited by the other daugher.
C. Vertical cleavage plane, where one daughter cell inherits both apical membrane and basal process.

Hatten, 1987). Quantification of this radial movement revealed a "saltatory pattern of advance" with a net average velocity of $33\pm20\mu m.hour^{-1}$, where the migration of the cell soma was punctuated with interludes of migratory arrest.

Modes of Radial Migration Revealed by Brain Slice Culture

In recent times the advent of brain slice culture has allowed the observation of populations of migrating cells, labelled with vital dyes, in a timelapse manner. The studies of Nadarajah and colleagues provided much insight into the mechanics of how neurons migrate in the embryonic mouse forebrain (Nadarajah et al., 2001 and Nadarajah et al., 2002). The authors describe two different modes of radial migration, which they labelled 'somal translocation' and 'glia-guided locomotion'. The former mode of migration apparently predominates in early corticogenesis, before radial glial networks are established. Migrating neurons maintain a basal contact with the pia throughout their migration and their cell body 'translocates' to more basal positions. Cells undergoing 'glia-guided locomotion' lose contact with both ventricular and pial surfaces, instead displaying short leading and trailing processes during their migration. Their migration in timelapse movies is saltatory, as described by the Hatten studies (Edmondson and Hatten, 1987 and Gasser and Hatten, 1990a and 1990b). However, the Nadarajah studies did not demonstrate glial processes and locomoting cells simultaneously. The authors speculate that the glial processes aid migration of later-born neurons since they must navigate an 'inside-out' path through to the cortical plate as the cortex develops in thickness and complexity later in development (see Nadarajah, 2003).

Modern Perspectives of Radial Migration

An interesting aspect of the modern radial glial literature is the disparity in the models of radial glial cell division as proposed by Noctor and colleagues (Noctor et al., 2001 and 2004) and Miyata and colleagues (Miyata et al., 2001). Both made timelapse movies of single fluorescent-labelled VZ radial glia in cortical slices to observe the asymmetric divisions of these cells. Despite using very similar methods, the two propose quite differing models for the mechanics of radial glial cell division. According to the Noctor model, following asymmetric radial glia-neuron division the radial glial daughter retains

the bipolar morphology and processes contacting both ventricle and pia. Miyata and colleagues report that asymmetric radial glial cell division results in the neuronal daughter inheriting the basal, pial-contacting process and the radial glial daughter retaining the ventricular process. The neuronal daughter uses this basal process to migrate into the cortical plate; the radial glial daughter regrows a new basal process. Although the optical quality of the Miyata study was not great enough to exactly see this sequence of events, the Noctor timelapses of 2004 certainly do not show anything like this asymmetric process inheritance. Rather, the asymmetric radial glial cell divisions observed by Noctor and colleagues appear to be more akin to a 'budding' process. How the two sets of authors came up with completely opposing models using almost exactly the same system remains something of a conundrum. One possible answer is that process inheritance is not stereotyped and is in fact a stochastic occurrence, with newborn neurons capable of radially migrating into the CP with or without a basal process; if this were the case, both groups of researchers would see the divisions they wanted to see.

Radial Migration In Vivo

An oft-overlooked point to remember is that radial migration has yet to be visualised *in vivo*, in an intact brain. Despite the importance of radial migration as a developmental process, many researchers appear content with the existing data from fixed tissue and culture systems. It is not known if the saltatory migration of cultured granule cells is actually real and characteristic of glia-guided migration, for example. What is the velocity of radial migration *in vivo*, where migrating neurons must negotiate a dense, three-dimensional territory, without being influenced by culture medium or other *in vitro* conditions? Another interesting question is the comparative aspect of radial migration: do teleost fish, with their simpler Type I brain organisation (Butler and Hodos, 1996) possess the same kinds of radial migration as have been described in the mouse brain? The zebrafish provides an excellent model for *in vivo* studies to address these questions.

Zebrafish as a Model for Studies of Neurogenesis

Teleostei is the name given to the superorder of those actinopterygian fish that possess a swim bladder. There have been well over 19,000 teleost species described to date,

making them by far the most successful vertebrate on earth; in contrast class *aves*, the next most numerous, contains approximately 9,000 species (McNeely et al., 1990). It is therefore surprising that only recently have teleost fish have been regarded as a model organism for vertebrate neuroscience research, when the majority of vertebrates on earth are in fact teleosts. However, as early as the 1970's George Streisinger identified the potential of a short generation time, prolific tropical fish as a model organism for studying developmental biology. Fish generally reproduce by external fertilisation and unlike marine or coldwater species that tend to be annual even in captivity, their tropical freshwater cousins are highly fecund in the aquarium, spawning frequently and with large numbers of offspring. Streisinger chose *Danio rerio* (originally described by Hamilton in 1882 as *Brachydanio rerio*, this species was reclassified following mitochondrial ribosomal analysis in 1993: see Meyer et al., 1993), the zebrafish, a cyprinid native to the Indian subcontinent already popular throughout the world as a decorative aquarium fish. The embryo of the zebrafish possesses many advantages for the researcher of early embryonic development. They are transparent, making them ideal for microscopy and live imaging. Fertilisation and development are external, clutch sizes of two to three hundred are common from a single mating and well-maintained pairs are capable of spawning once a week, providing large population numbers for the researcher. Embryonic development ends approximately three days after fertilisation with larval hatching, making studies of development very rapid. The utility of the zebrafish as a model for lineage studies of neurogenesis was demonstrated by the study of Lyons and colleagues (Lyons et al., 2003). Here the authors were able to follow the lineage of individual progenitors over multiple rounds of cell division in an unperturbed, intact vertebrate brain. In particular, the zebrafish hindbrain is readily accessible for single cell dye injection since the roofplate of this brain region is very thin and transparent. The Lyons study revealed much about progenitor cell lineage and division symmetry over the first two days of embryonic development. The authors also reported that although most hindbrain neurons are born by 48hpf, a small number of putative progenitors remain at this stage, and these cells are radial in morphology and express both zrf-1 and GFAP. A minority of these GFAP-expressing cells was also shown to express the marker of proliferation phosphorylated Histone-3 (H3p or PH3). The

possibility that these late-occurring hindbrain progenitors are a teleost equivalent of the neurogenic radial glia recently described in mammals is an intriguing one.

Aims

Our newfound knowledge of radial glia and their involvement in embryonic neurogenesis is almost entirely restricted to the mammalian telencephalon. With respect to these recent developments, this thesis is an investigation of radial glia and neurogenesis in not only a different vertebrate model organism, but in a different brain region, the rhombencephalon. This comparative analysis will broaden the scope of the field of neurogenic radial glial research considerably. The specific aims of this project are:

1. To characterise the progenitors that comprise the hindbrain ventricular zone during mid- to late neurogenesis (36hpf to 72hpf). In particular, the cellular composition of the glial curtain, a structure that appears to be unique to the zebrafish hindbrain, will be investigated. The correlation between glial fibrillary acidic protein (GFAP) expression and neurogenesis will be examined.
2. The developmental fate of late-occurring ventricular zone progenitors will be mapped using a combination of single-cell dye injection and single-cell electroporation techniques. The mode of progenitor cell division, and division orientation, will be recorded.
3. Using a stable GFP-expressing transgenic, the fate of a specific population of ventricular zone cells will be observed over embryonic and early larval stages. Timelapse movies will be recorded to observe the behaviour of these cells in the unperturbed, intact hindbrain; in particular, to examine the radial migration of newborn neurons during neurogenesis.

Chapter Two

General Methods & Materials

Many of the following methods are based on established protocols or are refinements of existing techniques, such as those published in The Zebrafish Book (Westerfeld, 1995). Where deemed necessary I have described the techniques in further detail in the methods section of the relevant results chapter.

1. *Animals*
2. *Embryo Culture*
3. *Immunohistochemistry*
4. *BrdU Incorporation and Visualisation*
5. *Histological Methods*
6. *Unbiased Stereology*
7. *Single Cell Dye Injection*
8. *7-Day Lineage Tracing by Electroporation*
9. *Microscopy*
10. *Imaging and Image Analysis*

1. Animals

Wild type or transgenic Tg(HuC:GFP) (Park et al, 2000) or Tg(zFoxD3:GFP) (Gilmour at al., 2002) zebrafish (*Danio rerio*) were maintained under standard conditions as described by Westerfeld (Westerfeld, 1995) on a 14-hour photoperiod in the UCL fish facility. Embryos were obtained by natural matings, incubated at 28°C and staged by hours post fertilisation (hpf) according to Kimmel and colleagues (Kimmel et al., 1995). All procedures were carried out in strict adherence to the terms of the 1986 Animals (Scientific Procedures) Act.

2. Embryo Culture

Embryos obtained from natural matings were placed in water drawn from the UCL fish facility to which methylene blue was added to reduce fungal infection and maintained at 28°C in a tissue culture incubator (Westerfeld, 1995). In some cases embryonic development was retarded or accelerated by decreasing or increasing the incubation

temperature to 22°C or 32°C respectively, as previously described (Kimmel et al., 1995). Prior to manipulation the embryos were dechorionated either mechanically with watchmaker's forceps or by treatment with 20mgml^{-1} Pronase (protease from *S. griseus*, Sigma) and the embryos placed in 'embryo medium', an aqueous mixed salt solution designed to replace the chorionic fluid (Westerfield, 1995). After 24 hours of development, approximately 0.003% phenylthiourea (PTU, Sigma) was added to the embryo medium to prevent pigment formation in the embryos; this has no reported adverse effects on the early development of the zebrafish (Westerfeld, 1995). During manipulation embryos were anaesthetised with MS-222 Tricaine (3-amino benzoic acid ethyl ester, Sigma), a local anaesthetic.

3. Immunohistochemistry

Embryos for immunohistochemistry were dechorionated, heavily anaesthetised with tricaine and fixed in 6% paraformaldehyde (PFA, Sigma) in PBS (phosphate-buffered saline, Sigma) overnight at 4°C. The primary antibodies used in this project are listed in Chapter Three (see Table 3.1).

Wholemount antibody staining was carried out according to fairly standard zebrafish protocols (see Westerfeld, 1995):

1. Remove from fixative and wash with PBS.
2. Dehydrate with a methanol-PBS series into 100% methanol, store at –20°C overnight.
3. Return to room temperature and rehydrate with a PBS-methanol series until the embryos are in 100% PBS.
4. For embryos older than 36hpf, embryos permeabilise with 0.25% trypsin (Sigma) on ice. (Embryos were generally treated for between 10 – 30 minutes).
5. Wash 3 x 5 minutes in PBS and refix with 5% PFA for 20 minutes at room temperature.
6. Wash off fixative with a 5-minute wash in PBS.
7. Place embryos in 3 x 5 minutes washes in PBTr (PBS with 1% Triton X-100 (Sigma))

8. Serum block with 10% normal goat serum (Sigma) in PBTr for one hour at room temperature on shaker.
9. Dilute antibodies in 10% serum and add to embryos. Incubate embryos at 4°C on a shaker overnight.
10. Return to room temperature and wash in PBTr on a shaker for four hours, changing the solution every 20 minutes. (Antibodies frequently used were recycled once after use).
11. Dilute secondary antibodies (species-specific fluorophore-conjugated antibodies (Alexa-488 (green) or Alexa-568 (red), Molecular Probes)) 1:200 in 10% serum and add to embryos. Incubate overnight at 4°C on shaker.
12. Return to room temperature and wash in PBTr on a shaker for four hours, changing the solution every 20 minutes.
13. Post-fix in 5% PFA for 20 minutes at room temperature.
14. Mount in 50% glycerol-PBS for imaging.

4. BrdU Incorporation and Visualisation

Pulse Labelling

1. Gradually bring embryos up to 15% DMSO (Sigma) in embryo medium on a shaker.
2. The final concentration of BrdU (Sigma) is $2mgml^{-1}$ in embryo medium in 15% DMSO. Add to embryos and place the Petri-dish on ice for 30 minutes.
3. Place back in embryo medium until fixation or further manipulation.

Visualisation of Incorporated BrdU

1. Fix in 4% PFA for at least 24h at 4°C.
2. Dehydrate embryos in 100% methanol for 1hr at –20°C.
3. Rehydrate embryos through PBS/methanol series.
4. Wash in PBS 2x 5 minutes.
5. Permeabilise embryos in 10 μgml^{-1} proteinase K (Sigma) for 20 – 40 minutes.
6. Rinse in glycine (2mg/ml) twice.
7. Rinse in PBS a few times.
8. Re-fix in 4%PFA for 30 minutes room temperature.
9. Wash in dH_2O a few times.

10. Incubate in 2N HCL for 1hr (make fresh each time: 8.6ml stock/50ml dH_20).
11. Wash in PBTr a few times.
12. Block in 2% normal goat serum for 1 hour.
13. Incubate in primary antibody (1:200) at least overnight.
14. Wash in PBTr 6-8 x 15 minutes.
15. Incubate in secondary antibody overnight.
16. Wash off secondary antibody.
17. Refix for about 24 hrs prior to dissection.

5. Histological Methods

The zebrafish embryo becomes very resistant to antibody penetration following approximately two days of development. To counter this it was found necessary to perform antibody staining on microtome sections of paraffin-embedded embryos rather than in wholemount preparations. Both paraffin embedding and PA-S reaction staining schedules are modifications of the methods described by various authors in Carleton's Histological Technique (Second Edition, Drury and Wallington, 1967).

Paraffin Embedding

1. Following deep anaesthesia with MS-222 Tricaine, fix the embryos in 5% PFA overnight at 4°C. Between thirty and one hundred embryos is ideal for one wax block.
2. Return to room temperature and wash 3 x 5 minutes in PBS in a glass bijou.
3. Dehydrate with a graded PBS-ethanol (50%, 70%, 95% and 100%) series into 100% ethanol.
4. Following 2 x 10 minutes in 100% ethanol, move embryos to 50% ethanol-Histoclear (Sigma) for 10 minutes
5. 2 x 20 minutes in 100% Histoclear.
6. Place embryos in Histoclear into 55°C oven for 30 minutes.
7. Add one drop of molten wax to the bijou and mix well. Add another drop and repeat. Continue to do this until the liquid in the bijou is approximately 50% paraffin wax. Leave in the oven for one hour.

8. Remove all the wax-Histoclear and add fresh paraffin wax. Stir well and leave for one hour.
9. Remove all the wax and add fresh wax. Mix and leave for one hour.
10. Agitate the contents of the bijou well to prevent embryos sticking to the bottom, and then pour into a plastic mould. Leave to set overnight.
11. Cut away the plastic mould and remove the wax block. If necessary trim the block to a suitable size with a scalpel and write the sample name on the side with a marker pen.
12. Cut 7μm sections on the microtome and mount sections onto Vectabond (Vector Labs, Burlingame) reagent-treated slides. Dry for twenty minutes in 55°C oven.

Harris' Haematoxylin

This recipe is according to Harris, 1900, with modifications by Mallory, 1938 (in Drury and Wallington, 1967). Although this form of haematoxylin is commonly used in exfoliative cytology and sex chromatin staining, I found it most suitable for nuclear staining of thin zebrafish sections.

1. Dissolve 20g potassium alum in 200ml hot distilled water.
2. Dissolve 1g haematoxylin in 10ml absolute alcohol and add this to the alum solution.
3. Bring the solution to the boil and add 0.5g mercuric oxide. The solution should turn dark purple.
4. Cool rapidly under the tap and then filter.
5. Add 8ml glacial acetic acid. Store at room temperature.

Schiff's Reagent

Given the unavailability of gaseous sulphur dioxide as called for by McManus, I used the recipe as described by Lillie, 1956 (in Drury and Wallington, 1967).

1. Boil 200ml of distilled water and add 1g of basic fuchsin. When dissolved, cool and filter.
2. Add 2g potassium metabisulfite and 20ml 1N HCl and stand overnight in the dark
3. Add 300mg of activated charcoal and stir with a magnetic stirrer bar for 5 minutes.
4. Filter; the filtrate should be clear and either colourless or with a pale straw tinge.
5. Store at 4°C.

Periodic Acid-Schiff Reaction Modified for Zebrafish Sections

This protocol is by McManus, 1946 and modified by Pearse, 1959 (in Drury and Wallington, 1967) but with modifications for the small size of zebrafish sections.

1. Dewax the sections by 2 x 10 minutes in Histoclear, then bring to water: 5 minutes each in a graded ethanol-water series (100% ethanol, 95%, 70%, 50% then water).
2. Oxidise for 5 minutes with 1% aqueous periodic acid.
3. Wash in running tap water for 5 minutes, then place in distilled water.
4. Place in Schiff's reagent for 20 minutes.
5. Rinse 3 x 10 minutes in freshly made up 0.5% aqueous sodium metabisulfite.
6. Wash in running tap water for ten minutes.
7. Stain nuclei with Harris' haematoxylin by dipping the slide once into the stain and then placing slides in acid alcohol.
8. Differentiate the haematoxylin in Scott's tap water for 10 minutes, then check the extent of staining under a dissection microscope. If the stain is too strong, then place slides back in acid alcohol, if too weak, dip once more in haematoxylin. Once the level of staining is deemed suitable, place the slides under running tap water for ten minutes.
9. Dehydrate the sections in a water-alcohol series and then clear in Histoclear and mount in DPX.

Protocol for Antibody Staining of Paraffin-Embedded Sections

1. Dewax the sections by 2 x 10 minutes in Histoclear, then bring to water then PBS: 5 minutes each in a graded ethanol-water series (100% ethanol, 95%, 70%, 50% then water, then 50% PBS then PBS).
2. Ring the sections with a wax pen (Dako) and then permeabilise with 10 minutes in ice-cold 1:1 methanol:acetone.
3. Wash slides in PBS, then into PBTr.
4. Serum block with 10% normal goat serum (Sigma) in PBTr for one hour at room temperature in a humid chamber.
5. Dilute antibodies in 10% serum and apply to sections. Incubate at 4°C overnight.

6. Return to room temperature and wash in PBTr for four hours, changing the solution every 20 minutes.
7. Dilute secondary antibodies (species-specific fluorophore-conjugated antibodies (Alexa-488 (green) or Alexa-568 (red), Molecular Probes)) 1:200 in 10% serum and apply to sections. Incubate overnight at 4°C.
8. Return to room temperature and wash in PBTr for four hours, changing the solution every 20 minutes.
9. Mount in 50% glycerol in PBS, seal with nail varnish and image.

6. Unbiased Stereology

The 'disector' method (Sterio, 1984) was used to quantify cells in a number of my experiments. This technique is a methodology for counting cell number in a 3D tissue in a stereologically based, unbiased fashion. Many journals currently require authors to use such techniques (see Saper, 1996). In our laboratory this method has been used successfully for a number of applications (see Lyons et al., 2003).

The specimen to be counted is imaged as a series of serial sections using laser scanning confocal microscopy. A critical factor in this method is using a step size smaller than the size of the objects being counted. Counting cell bodies or cell nuclei, a step size of 3μm was found to be sufficiently small for this purpose. The series is displayed in the program NIH Image and starting with the first section, each time the section transects a cell profile it is counted, so long as the same cell was not seen in the previous section. By this method each cell is only counted the first time it is encountered in the series. Live or fixed and immunostained embryos were imaged from the dorsal aspect by LSCM, and stacks of sections were saved as tagged-image file format (TIFF) files and imported into NIH Image for counting.

7. Single Cell Dye Injection

My technique for single cell dye injection is a personal modification of existing protocols developed in this laboratory (see Clarke, 1999).

Embryos

48hpf wild type or Tg(HuC:GFP) embryos were dechorionated, anaesthetised in MS-222 Tricaine and immobilised in 1.5% low melting point agarose (Sigma) in embryo medium. To facilitate access to the brain, the skin over the region of interest of the embryo was treated with $10mgml^{-1}$ Pronase (protease from *S. griseus*, Sigma) for exactly two minutes before the dye labelling procedure.

Microelectrodes

Microelectrodes were prepared from thin-walled 1.2mm OD aluminosilicate glass with internal filament (A-M Systems, Everett, WA) using a P-87 Flaming-Brown micropipette puller (Sutter, Novarto, Califonia). The microelectrode was designed to be very sharp to ensure the chance of labelling a single cell and when filled, to have a resistance of approximately 150 megaohms. When examined under 400x magnification with DIC optics the tip should not have a 'ribbed' or banded appearance but should come to a sharp, invisibly small tip with a blue-coloured taper.

Microelectrodes were backfilled with a small amount of 5% 3,000 Mw biotin- and TRITC-conjugated dextran (Micro-Ruby™, Molecular Probes, Oregon) in sterile-filtered distilled water and then approximately 50µl 1M KCl immediately prior to use. I found that the Micro-Ruby™ was preferable to some of the other dextrans on offer in that it did not clog the microelectrode as easily even at high concentrations and had more favourable electrophoretic properties.

Iontophoresis

Single radial glial cells in the hindbrain VZ were labelled by low amplitude current injection using a Neurolog amplifier (Digitimer). After advancing the microelectrode tip until it came to rest against the ventricular surface of the brain, a brief hyperpolarisation of approximately one second was sufficient to label a single cell. The current amplitude was between 2 – 8nA.

Success of injection was monitored by epifluorescence microscopy. Following dye labelling, the injected cell was examined under x400 magnification on a Zeiss Axioplan 2 microscope to ensure that no more than one cell was labelled and that the single cell was healthy. The embryo was immediately discounted from the study if the labelled cell was obviously extruded from the neuroepithelium or where more than one cell was labelled. Where successful the single cell was photographed and the embryos incubated in embryo medium at 28°C for 24 hours. After this period the embryos were photographed again and the labelled clone phenotyped according to morphology alone or by morphology and GFP expression in the Tg(HuC:GFP) transgenic line.

8. Single-Cell Electroporation

Electroporation was performed according to a technique based upon the protocol first described by Haas and colleagues (Haas et al., 2001) and modified in the Clarke lab by Marcel Tawk (see Concha et al., 2003). For both single cell labelling by intracellular dextran injection and electroporation a Nikon Optiphot fixed stage epifluorescence microscope with a 40x dry objective lens was used. The microscope was mounted on an air table to negate background vibration and stable micromanipulators with micrometer resolution were used to target cells; this proved to be crucial when carrying out single cell injections.

Embryos

48hpf wild type or HuC::GFP embryos were dechorionated, anaesthetised in MS-222 Tricaine and mounted in 1.5% low melting point agarose (Sigma) to hold the embryo in place inside a chamber device to facilitate embryo orientation and manipulation during the electroporation procedure. To facilitate access to the brain, the agarose covering the part of the embryo containing the posterior brain was removed and the skin in this region of interest was treated with 20mgml^{-1} Pronase (protease from *S. griseus*, Sigma) for exactly two minutes before the electroporation procedure.

Microelectrodes for Electroporation

Microelectrodes were prepared from thin-walled 1.2mm OD aluminosilicate glass with internal filament (A-M Systems, Everett) using a P-87 Flaming-Brown micropipette

puller (Sutter, Novarto). The microelectrode was designed to have a patch-like tip approximately three to five microns in diameter and a fairly steep shoulder, which was confirmed by x400 DIC light microscopy.

Electroporation

The tip of the electroporation microelectrode was advanced towards the ventricular surface of the hindbrain. Once the tip was in contact with the brain, short pulses of voltage were applied across the embryo. In addition to varying the voltage, the number and duration of pulses was changed until a suitable schedule was determined. For 48hpf embryos, three 1-second trains of 200Hz pulses at 25V proved sufficient for reproducible single-cell electroporation.

Following electroporation the embryo was recovered from the agarose and allowed to develop in embryo medium in a 28°C incubator. The following day embryos were checked to determine if single cell electroporation was successful. Those embryos that possessed single, GFP-expressing cells were imaged by epifluorescence or LSCM and replaced in the incubator. Seven days post-electroporation the GFP-expressing cells were imaged by LSCM.

9. Microscopy

Embryo sorting and staging was carried out on a Nikon SMZU dissecting microscope. Single cell labelling was performed on a Nikon Optiphot fixed-stage epifluorescence microscope with a 40x dry objective lens. The microscope was mounted on an air table to negate background vibration; this proved to be crucial when carrying out single cell injections. Immunostained, dye-injected or electroporated embryos were generally imaged by standard epifluorescence microscopy using a Zeiss Axioplan 2 microscope and 40x or 63x water immersion objective lenses. A Leica laser scanning confocal microscope (Leica Microsystems, Heidelberg) was used to analyse certain fluorescent specimens such as those that could not be resolved by epifluorescence.

10. Imaging and Image Analysis

The Zeiss Axioplan 2 microscope was linked to a Hamamatsu ORCA ER CCD camera and controlled using the programme 'Openlab' (Version 3.1.5, Improvision). This

software made possible automated z-series image capture and accumulation, volume deconvolution and timelapse microscopy. Single images from Openlab were transferred as Tagged-Image File Format (TIFF) files and imported into Adobe Photoshop (Version 6.0 or 7.0, Adobe Systems) for publishing purposes. Timelapse videos were constructed from frames captured in Openlab and then saved as movies in Quicktime format.

Confocal scans and maximum intensity projections were captured on the confocal Leica microscope using Leica TCS NT software (Version 1.6.587) saved in Leica software format and then subsequently analysed by NIH Image and Image J programs. Single confocal sections or projections were then imported into Adobe Photoshop for publishing purposes.

Chapter Three

Characterisation of Ventricular Zone Progenitors during Hindbrain Neurogenesis

Introduction

As embryonic neurogenesis progresses, the cells comprising the neural tube become divided into two main compartments: the ventricular zone (VZ), containing undifferentiated, proliferative progenitors, and the mantle zone (MZ), made up of postmitotic neurons. During early neurogenesis, the progenitors of the developing brain are exclusively neuroepithelial cells, the least differentiated and most primitive neural cell type. However, as neurogenesis proceeds the VZ population becomes more complex. In addition to progenitor cell types such as neuroepithelial cells, there are also radial glia, newborn neurons and various transitional forms. The exact composition of the VZ remains the subject of debate, and experimental evidence is conflicting. Early clonal analyses indicated that the VZ was populated by a heterogeneous mixture of progenitors, with cells largely restricted to single lineages (Price and Thurlow, 1988, Luskin et al., 1988 and Grove et al., 1993). Later studies showed that a small minority of VZ cells was multipotential, giving rise to neurons, oligodendrocytes and astrocytes (Williams and Price, 1995). In contrast, a number of recent papers have indicated, through a combination of clonal analysis, timelapse microscopy and electrophysiological recordings, that the cortical VZ may be almost completely composed of a single type of multipotent progenitor, the radial glia (Noctor et al., 2001, 2002 and 2004). However, the study of Hartfuss and colleagues has indicated that a subpopulation of proliferating cells that does not express the radial glial markers RC2, GLAST or BLBP exists in the E14 mouse cortex (Hartfuss et al., 2001), showing that non-radial glial progenitors are also present in the VZ at this stage.

In contrast with the mammalian brain, far fewer studies have analysed the composition of the VZ in fishes, however, the zebrafish hindbrain may be advantageous for such studies due to its far smaller total cell number and comparative simplicity. Lyons and colleagues describe how at 48hpf, many cells of the zebrafish hindbrain VZ express GFAP, and a minority of these cells express the marker of proliferation PH3 (Lyons et al., 2003). Whether all the cells of the zebrafish hindbrain VZ are radial glia, as the Noctor studies suggest, or the VZ is a heterogeneous mixture of radial glia, non-radial

glial progenitors and other cell types as older literature describes, will be tested in this chapter.

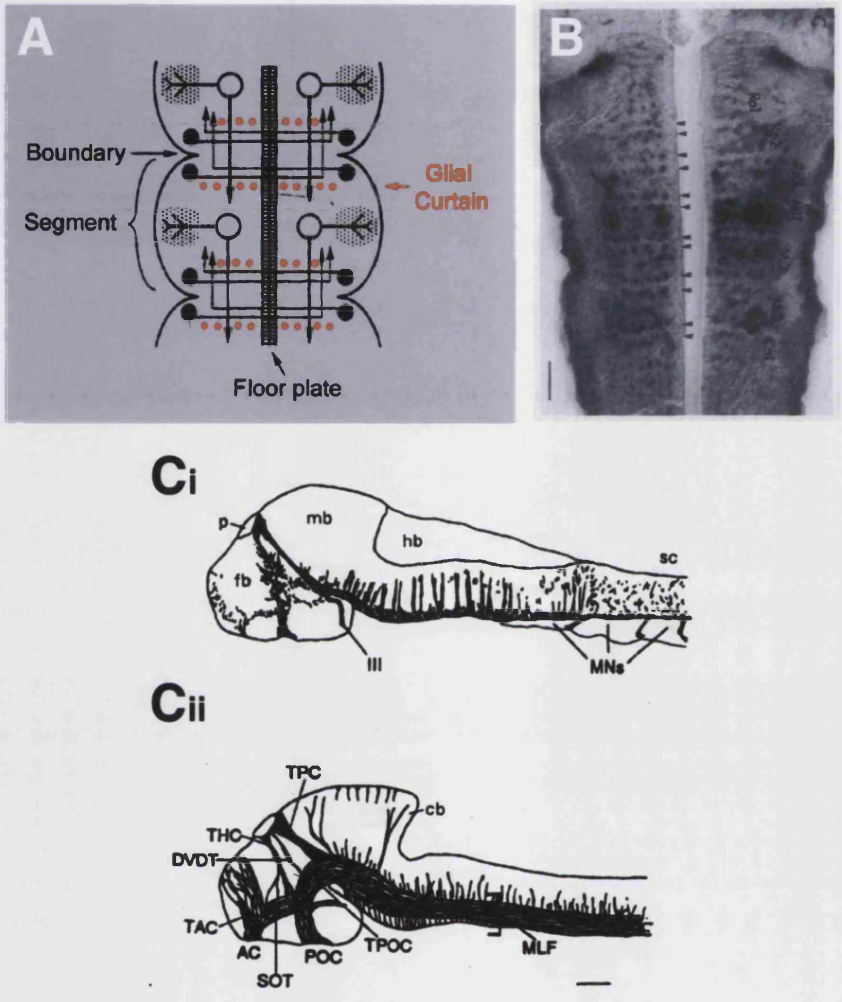
In the fish CNS, radial glia were first reported, using the Golgi method, by von Lenhossék in sharks (von Lenhossék, 1892) and in teleost fishes, by Retzius and van Gehuchten (Retzius, 1893 and van Gehuchten, 1894). However, labelling radial glia with GFAP in fishes has produced mixed results. In modern times, it has been shown that commercial antibodies to mammalian GFAP cross-react with many fish species, although the GFAP antigen is complex in teleosts. In mammals GFAP is isolated as an acidic 50kDa protein (Eng et al., 1971 and Dahl and Bignami, 1985) yet the commercially available anti-GFAP antibody raised to the bovine protein binds at least five intermediate filament antigens 48kDa, 50kDa, 52kDa 58kDa and 80kDa in size in the goldfish brain (Levine, 1989). Of these, the 58- and 80kDa-sized proteins appear specific to astrocytes in the optic nerve whilst the 48kDa and 50kDa species appear more prevalent in the rest of the brain in cells and processes with a radial phenotype (Levine, 1989). An antibody raised to goldfish GFAP by Nona and colleagues (Nona et al., 1989) was subsequently shown to bind two species, 45kDa and 51kDa, in zebrafish (Marcus and Easter, 1995) and these are probably homologues of the goldfish protein. Therefore compared to the other two main cytoskeletal building components, tubulin microtubules and actin microfilaments, which are both highly conserved across all chordates, chondroichthyeans and osteichthyeans demonstrate variability in GFAP intermediate filaments when compared to other vertebrate classes (Dahl et al., 1985).
Kálmán reports that immunostaining with antibodies to GFAP alone is insufficient to completely realise radial glial networks since very fine glial processes are undetectable by immunohistochemistry and must be determined by traditional silver or gold impregnation techniques (Kálmán, 1998). Golgi staining in the trout forebrain shows some radial glia possessing processes with plentiful spiny elaborations and filopodia (van Gehuchten, 1894) and Stensaas and Stensaas report that the radial glia of the pigeon forebrain stained by the same technique possess '...distal processes covered by innumerable, fine, lamellate excrescences' (Stensaas and Stensaas, 1968). GFAP antibody stains of radial glia in both fish and birds do not show these elaborations to the same extent, suggesting that the very small extensions and filopodia of the radial glial

process owe their structure to cytoskeletal components other than GFAP. Similar findings have been reported in the macaque (Levitt and Rakic, 1980) and intracellular fluorescent dextran labelling reveals the same 'spiny processes' in radial glia of the turtle forebrain (Weissman et al., 2003).

The need for an exclusive marker for teleost radial glia appeared to be finally satisfied by the efforts of a research group at Okayama University in February 2000. Tomizawa and colleagues laid claim to having isolated a specific marker of teleost radial glia, publishing their monoclonal antibody C-4 that apparently stains such cells in the adult zebrafish CNS (Tomizawa et al., 2000). However, the examples of C-4 staining the authors show in their study are not convincing of radial glia and despite my many attempts to contact them in order to obtain an aliquot of this antibody for my own immunostaining, they did not reply to me at all, or indeed to any other research group that also wished to test C-4 in their own laboratory (J. Scholes, personal communication). Therefore without the antibody C-4, in this study I will use antibodies to GFAP to reveal radial glia-like cells in the hindbrain VZ. It has already been shown that many progenitors in the 36hpf zebrafish hindbrain express GFAP (Lyons et al., 2003) but a more detailed description of the temporal expression of this intermediate filament is required.

Bill Trevarrow and colleagues first identified a putative early glial population in the hindbrain of the embryonic zebrafish over ten years ago (Trevarrow et al., 1990). By raising a panel of monoclonal antibodies to extracts of cytoskeletal or membrane preparations derived from CNS tissue, they described three populations of cells that appear in a stereotyped, repeating pattern (Figure 3.1) that corresponded to the rhombomeres of the hindbrain. The three populations were commissural interneurons, reticulospinal neurons and a third group they dubbed 'glial curtain.' The glial curtain was described as a fan- or curtain-like structure of glial processes aligned along the dorsoventral axis that is present at either side of each rhombomere boundary (Figure 3.1). It appears the authors described this as a 'glial' curtain because they did not identify any neuronal component of the structure. Five years later, Marcus and Easter reported that the glial curtain is also labelled by antibodies to GFAP (Figure 3.1) (Marcus and Easter, 1995). The authors also showed that in the glial curtain, there

Figure 3.1. Rhombomeric organisation and marker expression within the embryonic zebrafish hindbrain.
Trevarrow and colleagues raised a panel of monoclonal antibodies to CNS protein extracts and identified three distinct cell populations within the hindbrain (A and B).
A. Schematic diagram adapted from Trevarrow et al, 1990, showing reticulospinal neurons (open circles) found only in rhombomere centres, commissural interneurons (filled circles) found only in specialised boundary regions and the 'glial curtains' (red) lying either side of the segment boundaries. These three cell populations are found in a stereotyped organisation that reiterates the rhombomeric segments.
B. Photomicrograph adapted from the same study. 36hpf hindbrain viewed from the dorsal aspect. The authors' monoclonal antibody zrf-1 preferentially stains the glial curtains (arrowheads). Some reticulospinal neurons between the glial curtains have also been retrogradely labelled with HRP.
C. Low magnification *camera lucida* drawings adapted from Marcus and Easter, 1995, showing marker expression in the CNS: glial processes as revealed by GFAP (Ci) and axons labelled by HNK-1 (Cii). Some of the early axon tracts are also GFAP-positive, as are fibres projecting in the D-V axis in the hindbrain; these are the glial curtains. There are also HNK-1-positive processes contributing to the glial curtains, although the authors did not perform a simultaneous demonstration of the two antigens.


A
Boundary
Segment
Glial
Curtain
Floor plate
B
Ci
p
mb
hb
sc
fb
III
MNs
Cii
TPC
cb
THC
DVDT
TAC
AC
POC
TPOC
MLF
SOT

appeared to be an overlap in immunopositive staining between GFAP and the axonal marker HNK-1, although they did not attempt to label with the two antigens simultaneously (Figure 3.1). Despite its unusual and possibly unique nature, in neither the Trevarrow paper nor the Marcus and Easter study did the authors speculate on the possible role or function of the glial curtain. More recently, Lyons and colleagues showed that the glial curtains were composed of the basal processes of GFAP-expressing cells in the VZ (Lyons et al., 2003). They also showed that a minority of these GFAP-positive cells expressed the proliferative marker PH3. Therefore the identification of GFAP-expressing proliferative cells residing in the VZ is a most interesting development, from which two obvious questions arise: are these cells zebrafish radial glia, and are these radial glia-like cells neurogenic? By using marker expression and BrdU incorporation techniques in the Tg(HuC:GFP) transgenic line, I will attempt to address these questions.

In the foetal macaque CNS, Rakic described radial glia as forming a guiding rail or scaffold for the radial migration of newborn neurons (Rakic, 1971a, b and 1972). It was also later demonstrated that the radial processes of these radial glia expressed GFAP (Levitt and Rakic, 1980). Therefore there exists the possibility that in the zebrafish hindbrain the glial curtain, comprised of GFAP-expressing processes, may be acting in a similar fashion, with newborn neurons migrating along the fan- or curtain-like sheet of glial processes, out of the VZ and into the MZ. Simultaneous antibody staining to both GFAP and neuronal markers may reveal evidence for this kind of migratory behaviour.

The aim of this chapter is to characterise the cells that comprise the zebrafish hindbrain ventricular zone during late neurogenesis, using marker expression. In particular, the expression of the radial glial marker GFAP and the composition of the glial curtains will be examined in detail. The proliferation of GFAP-expressing VZ progenitors will be quantified, at the population level, from mid-neurogenesis (36hpf) to early larval stages by BrdU incorporation analysis.

Methods

Immunohistochemistry

Immunostaining was performed using the protocols described in Chapter Two. The primary antisera used are summarised in Table 3.1. In particular, I must acknowledge the services of the Developmental Studies Hybridoma Bank of Iowa University, and I am also obliged to John Scholes, for generously providing me with a kind gift of considerable quantities of the anti-GFAP antibody (Nona et al., 1985), and to Nigel Pringle, for the gift of the anti-GLAST antibody. The others were commercially available and their sources are as listed (Table 3.1).

DiI labelling

To observe their morphology cells in the VZ of the hindbrain were labelled with the lipophilic dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, Oregon) by either iontophoresis or mechanical application of solid dye crystals to the surface of the brain using a sharpened tungsten needle, on a Nikon Optiphot fluorescence dissecting microscope. For iontophoresis, DiI was made up to a concentration of $2mg.ml^{-1}$ in 95% ethanol, 5% dimethyl formamide and backfilled into a glass microelectrode specifically designed for iontophoresis of lipophilic dye. The DiI-loaded electrode was then backfilled with 100% ethanol and 2M LiCl and connected to a 12V battery, with current controlled by manual completion of the circuit by touching the negative electrode to the battery.

BrdU Incorporation Studies

BrdU incorporation studies were performed as described in the General Methods chapter. In general, the animals were sacrificed by deep anaesthesia with MS-222 tricaine and then fixed in 5% PFA 30 minutes after the BrdU pulse. Incorporated DNA analogue was detected by immunohistochemistry.

Laser Scanning Confocal Microscopy

LSCM was carried out using a fixed-stage Leica confocal microscope with 20x, 40x and 63x water immersion objective lenses. Images were acquired with Leica TCS

Antibody Name	Clone name	Raised in	Dilution	Source	Reference
anti-GFAP	polyclonal	rabbit	1:100	Gift, John Scholes	Nona et al, 1985
anti-tubulin	6-11B-1	mouse	1:200	SIGMA	LeDizet and Piperno, 1991
zrf-1	zrf-1	mouse	1:200	University of Oregon	Trevarrow et al, 1990
anti-PH3	polyclonal	rabbit	1:200	Upstate	Chadee et al, 1995
anti-BrdU	BU 33	mouse	1:200	SIGMA	Gratzner, 1982
anti-GFP	polyclonal	rabbit	1:1000	AMS Biotechnology	Chalfie et al, 1994
anti-HuC/D	16A11	mouse	1:50	Molecular Probes	Brashear et al, 1991
anti-GLAST	polyclonal	guinea pig	1:100	Gift, Nigel Pringle	Suarez et al, 2000
anti-vimentin	3B4	mouse	1:100	Dako	Azumi and Battifora, 1987
anti-S100	polyclonal	rabbit	1:100	Dako	Orchard, 2000
anti-vimentin	40E-C	mouse	1:100	DS Hybridoma Bank	Alvarez-Buylla et al, 1987
anti-nestin	Rat 401	mouse	1:100	DS Hybridoma Bank	Hockfield and McKay, 1981
anti-RC2	RC2	mouse	1:100	DS Hybridoma Bank	Misson et al, 1988

Table 3.1. Antibodies used in this study

Table 3.1 lists all the primary antibodies used in this study, with their sources, references and working dilutions.

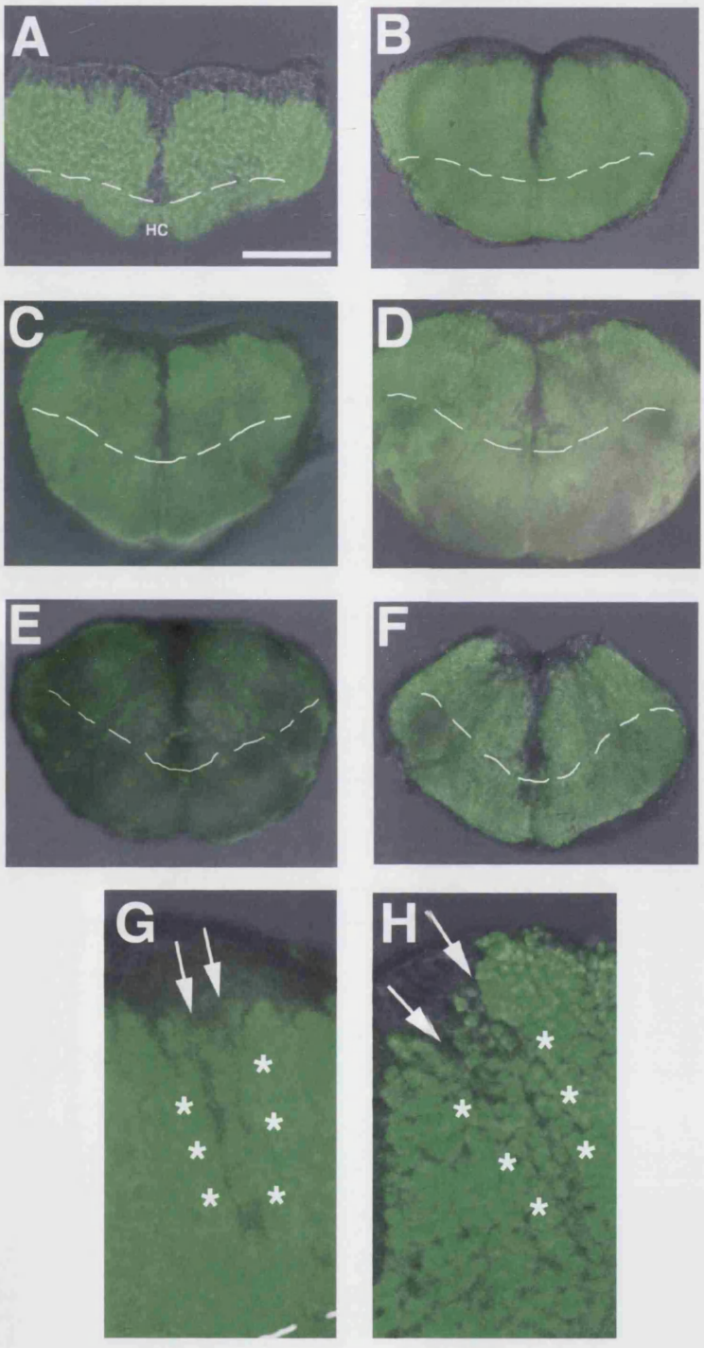
NTsoftware (Version 1.6.587, Leica Microsystems, Heidelberg). Captured confocal scans were saved as single tagged-image format files (TIFF) which were then exported to other computers for processing and analysis using NIH Image 1.63, Image J and Adobe Photoshop.

Results

The Hindbrain Ventricular Zone (VZ) as Revealed by the Tg(HuC:GFP) Transgenic Line Diminishes in Size as Neurogenesis Proceeds

All neurons in the Tg(HuC:GFP) transgenic line (Park et al, 2000) express GFP as early as 4 hours after their birth (Lyons et al 2003). This line has been invaluable not only to help phenotype terminally differentiated neurons but also to understand the overall organisation of the zebrafish hindbrain. Gross microscopic observation of transverse sections of the Tg(HuC:GFP) hindbrain reveals the VZ as a GFP-negative zone that decreases in size through embryonic development as the pool of VZ progenitors is depleted and post-mitotic neurons are added to the mantle zone (Figure 3.2). As morphogenesis proceeds between 24hpf and 48hpf, the hindbrain has the appearance of 'opening' outward as the two ventral halves of the neural tube close together at the midline while the dorsal halves flatten outwards as the roofplate expands and thins greatly. The dorsal VZ thus forms a flat horizontal floor to the fourth ventricle (Figure 3.2). By 48hpf the VZ is very distinct as a T-shaped motif of GFP-negative progenitors. The VZ can be characterised as two compartments, a dorsal horizontal zone and a medioventral zone of GFP-negative progenitors. It is likely that the medioventral domain is also the site of oligodendrogenesis although both domains support neurogenesis throughout late embryonic development. According to previous work, the majority of hindbrain neurogenesis is complete by 72hpf (Lyons et al., 2003) and the VZ is greatly reduced by this stage, although GFP-negative progenitors are still easily detected (Figure 3.2, B). This is also the stage at which the zebrafish hatches, and so embryonic development is considered complete and subsequent development referred to as 'larval.' At this hatching stage, GFP-negative cells are still detectable as the VZ is restricted to a thin, flattened domain approximately two to three cell body thicknesses wide that covers the dorsal surface of the hindbrain.

Figure 3.2. The hindbrain ventricular zone (VZ) revealed by the Tg(HuC:GFP) transgenic line during embryonic and larval development.
Maximum intensity projections of hindbrain transverse sections imaged by LSCM. Fluorescence is projected onto transmitted light. The broken white line delineates the approximate limit of the white matter of the marginal zone.
A. 48hpf. The VZ is distinguishable as a T-shaped domain of GFP-negative cells covering the dorsal and medial regions of the neural tube (arrows). A GFP-positive axon tract of the hindbrain commissures gives the appearance of a 'bridge' connecting the two halves of the hindbrain (HC).
B. 72hpf. The VZ is greatly diminished by this stage, comprising only two or three cell body-diameters at the dorsal extreme of the hindbrain, and a small group of cells clustered about the midline. The latter domain is also the site of oligodendrogenesis.
C. 96hpf. The expansion of the axon tracts in the marginal zone increases the DV dimension of the hindbrain, and although the VZ retains its T-shaped pattern, it is reduced to the thickness of approximately one or two cells. The GFP-negative domain in the very centre of the neural tube represents the likely region of oligodendrogenesis.
D. 120hpf. The white matter expands dorsolaterally as the longitudinal axon tracts continue to increase in width. The VZ becomes divided into two: dorsally, into a narrow box-like domain that lies atop the dorsal midline and ventrally, in a cluster of GFP-negative cells. Thin GFP-negative streaks emanating from the dorsal VZ and projecting into the mantle layer are clearly discernible (see G and H).
E. 7dpf. The dorsal VZ becomes compressed laterally, creating the appearance of an inverted triangle or Y-shaped domain.
F. 14dpf. The Y-shape of the VZ persists even two weeks after fertilisation, with the GFP-negative radial processes permeating the mantle layer still visible. The VZ has also thickened several cell diameters, implying an expansion of the progenitor cell compartment, over ten days after embryogenesis, and embryonic neurogenesis, has been completed.
G and H are single z-sections taken from the confocal z-series in B and E respectively.
G. At 72hpf, HuC:GFP-negative streaks are visible emanating from the dorsal VZ (arrows) and projecting radially through the mantle zone (asterisks). They most likely represent the location of basal processes of VZ progenitors, bundled together into thick fibres.
H. 7dpf. These GFP-negative streaks persist well into larval stages.
Scale bar: 100μm.


A
HC
B
C
D
E
F
G
H

By 7dpf the VZ has receded both laterally and ventrally, and remains as a distinct box-like domain straddling the dorsal midline (Figure 3.2, E). Tapered GFP-negative streaks are visible emanating from this area radially into the MZ (Figure 3.2, G and H) which are probably composed of the basal processes belonging to cells in the VZ. By this stage, over 50% of the transverse area of the hindbrain is comprised of the rapidly expanding marginal zone (Figure 3.2, E and F).

Cells Comprising the Hindbrain VZ Possess a Radial Morphology

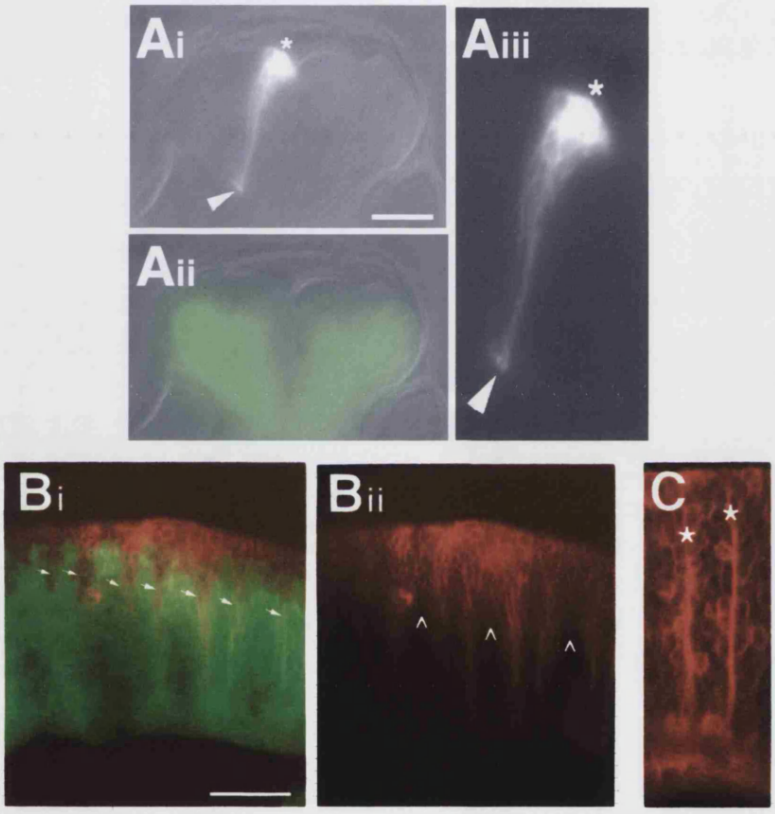
Labelling cells by iontophoresis with the lipophilic dye DiI reveals that the large majority of cells in the hindbrain VZ possess a radial morphology with their cell somata in the VZ (Figure 3.3, A). Cells at the dorsalmost margin of the hindbrain have no apparent ventricular process since their soma lies directly in contact with the ventricular space and have the appearance of being unipolar. Staining the ventricular surface with the lipophilic dye DiI reveals that all of the cells of the VZ preferentially extend their pial processes into bundles of radial fibres on either side of the rhombomere boundaries (Figure 3.3, B). These bundles are the glial curtains described in the literature previously (Trevarrow et al., 1990, Marcus and Easter, 1995 and Lyons et al., 2003). Fibres labelled with DiI project into these 'para'-boundary bundles regardless of the A-P position of their parent cell bodies within each hindbrain rhombomere, giving the appearance of arches of cells connecting to the fibres below them when viewed from the lateral aspect (Figure 3.3, B). When all D-V levels of the hindbrain are labelled with DiI, cell bodies at even very basal levels are seen to be in close proximity with the bundles of the glial curtains (Figure 3.3, C).

GFAP Expression in the Embryonic Hindbrain

The distribution and temporal expression of glial fibrillary acidic protein (GFAP) was analysed. A polyclonal antibody raised against goldfish GFAP (Nona et al., 1985) in rabbit was used for immunostaining on either sections or wholemount zebrafish. This antiserum has been shown to cross-react in Western blot and wholemount immunohistochemistry with the zebrafish protein (Marcus and Easter, 1995).

Although it has been previously described that GFAP is detectable as early as 15hpf in the zebrafish (Marcus and Easter, 1995), by my method I was unable to reliably detect

Figure 3.3. Morphology and patterning of cells in the hindbrain ventricular zone
A. Application of a crystal of lipophilic dye DiI to the ventricular surface of the hindbrain (asterisk) of a HuC::GFP transgenic zebrafish reveals cell bodies in the ventricular zone (VZ) projecting radial processes to the ventral pial extreme of the neural tube.
Ai Transverse section imaged by epifluorescence, with fluorescent signal superimposed over DIC transmitted light image. The processes belonging to VZ cells reach the pial surface of the hindbrain (arrowhead) and terminate in enlarged endfeet.
Aii. GFP-expressing postmitotic neurons show the extent of the mantle zone at this stage. Aiii. At higher magnification, the rounded, enlarged endfeet belonging to the pial processes of VZ cells are clearly visible.
Bi. DiI iontophoresed onto the ventricular surface of the hindbrain reveals the cells of the VZ preferentially project their processes into the glial curtains (arrows).
Bii Same image with red channel only, showing the VZ cells projecting their processes into the glial curtains appearing as 'arches' that correspond to the rhombomeric segmentation (inverted chevrons). Both images are a single confocal section of a z-series imaged by LSCM.
C. DiI applied to the ventricular surface and allowed to spread over 24 hours reveals the glial curtains as thick, fasciculated bundles (asterisks), to which cells in the mantle zone are also closely attached.
Scale bar: 100μm.


Ai
Aii
Aiii
Bi
Bii
C

the protein until 24hpf. At this stage, when viewed in transverse section, immunopositivity is localised to the pial endfeet of neuroepithelial (NE) cells and occasional cell bodies at the ventricular surface (Figure 3.4, A). By 36hpf, the cell bodies comprising the VZ express GFAP and the protein is absent in cell bodies in the mantle zone (Figure 3.4, B). The basal processes of VZ cells also express GFAP, and these processes fasciculate as they extend ventrally to form thick bundles or fibres (Figure 3.4, B – D). These bundles penetrate the thickness of the developing marginal zone and terminate in groups of endfeet in contact with the pial surface (Figure 3.4, B – D). GFAP immunoreactivity in the embryonic hindbrain peaks at approximately 36hpf, when virtually all the cells in the hindbrain VZ express GFAP, both in processes and cell bodies. By 48hpf, GFAP expression begins to decline in the cell bodies in the VZ and also in the more lateral basal fibres (Figure 3.4, D).

From the dorsal aspect it is apparent that a minority of cell bodies express the antigen to a higher degree than their neighbours, although all the cells that comprise the VZ express GFAP (Figure 3.4, I). The 'glial curtains' stain prominently for GFAP, although by my method the appearance of the curtains is one of repeating horseshoe-shaped loops with their open face at the midline rather than simple transverse rows of cells as first described by Trevarrow and colleagues (Figure 3.4, Iii). These loops iterate rhombomeres 2 to 6 (Figure 3.4, Iii). When viewed from the lateral aspect, the fibres and processes of the cells in the VZ are arranged in accordance with the rhombomeric units, with occasional fibres permeating the centres of each rhombomere but conspicuously absent from the specialised boundary regions between them (Figure 3.4, G).

At 72hpf GFAP immunoreactivity is practically undetectable in the hindbrain VZ, although staining remains strong in radial glia-like cells in the spinal cord, which serve as an internal control for antibody staining. However, by 120hpf a tiny group of GFAP-positive cell bodies in the VZ at the dorsal midline is immunopositive, as are strongly stained radial processes extending to the pia (Figure 3.4, E). There are also numerous weakly staining distal processes and rounded endfeet, whose cell bodies do not express GFAP. On reaching the interface of the grey and white matter, these processes branch in a highly complex fashion and permeate and invade the axons of the marginal zone in a manner highly reminiscent of radial glia described in classical literature. There is strong

Figure 3.4. Temporal expression of glial fibrillary acidic protein (GFAP) in the developing hindbrain

Immunostaining for GFAP in wildtype embryos. A – F are transverse sections, G and H are viewed from the lateral aspect, with anterior to the left and I and J are dorsal views, with anterior to the top.

A. 24hpf. Faint immunoreactivity is detectable at this stage in the majority of cells of the neural tube, with protein concentrated in endfeet at the pial sruface, although a minority of cell bodies rounded up at the ventricular surface stain strongly for GFAP, a feature that persists throughout embryonic development.

B. 36hpf. Glial processes stain very prominently for GFAP, especially at the pial surface where the fibres conjoin in the developing white matter. Cell bodies in the dorsal ventricular zone express GFAP, and it is the fasciculating processes of these cells, projecting to the pial surface, that comprise the glial curtains. Tangential fibres or processes crossing the ventral midline by this stage become visible (asterisks), which correspond to the developing hindbrain commissures. The positive immunoreactivity of these processes is most likely due to GFAP protein lingering in the cytoplasm of recently differentiated neurons.

C. 42hpf. As neurogenesis proceeds GFAP expression declines, especially in lateral areas of the hindbrain. The fibres of the glial curtains appear to divide and demarcate the axon tracts as they form in the developing white matter.

D. 48hpf. GFAP expression becomes more restricted to the pial endfeet and marginal layer, with GFAP-expressing cell bodies in the dorsal VZ less conspicuous, although occasional strongly expressing, rounded up cell bodies persist. By 72hpf, GFAP expression is essentially undetectable in the hindbrain.

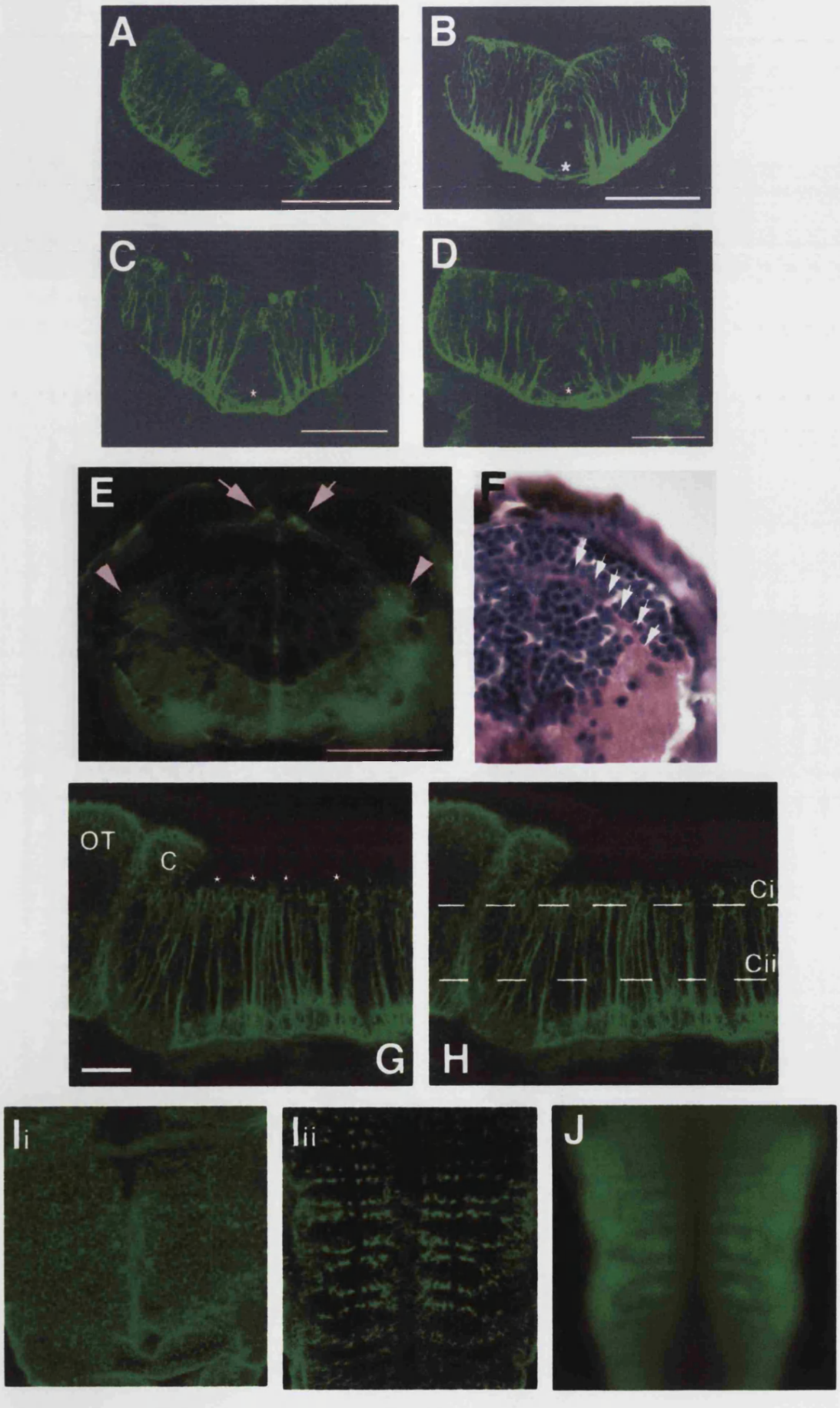
E. 7μm paraffin section of 5dpf hindbrain, immunostained for GFAP and imaged by epifluorescence. At this stage GFAP expression is restricted to a small number of cell bodies in the VZ (arrows) that project radial processes through the mantle layer and marginal zone and terminate in endfeet contacting the glia limitans. On reaching the marginal zone, these processes become branched and complex (arrowheads). There are also numerous GFAP-positive endfeet present at the circumference of the marginal zone, but their cell bodies do not express the protein, and also faint-staining radial fibres. There is widespread and punctate immunoreactivity throughout the marginal zone, and also strong staining in the floorplate and the major longitudinal axon fasciculi.

F. At 6dpf periodic acid-Schiff reaction in 7μm paraffin section reveals radial processes rich in glycogen granules (arrows) a feature indicative of radial glia.

G. 36hpf. Maximum intensity projection of wildtype embryo imaged from the lateral aspect by LSCM, anterior is to the left; GFAP expression reiterated the segmental rhombomeric pattern, with GFAP conspicuously absent from boundary regions (asterisks). Optic tectum (OC) and cerebellum (C).

H. Same image as A with broken lines Ci and Cii, representing levels of confocal sections shown in C.

I. GFAP expression in 36hpf hindbrain imaged from the dorsal aspect by LSCM; anterior is to the top. Both Ci and Cii are confocal sections of the same specimen. Ii. At the level of the ventricular zone (VZ), all cells express GFAP, with a minority expressing the protein to a higher degree than their neighbours. Iii. At the


A
B
C
D
E
F
OT
C
G
H
Ci
Cii
Ii
Iii
J

more ventral level, the glial curtains are apparent as GFAP-positive repeating, horseshoe-shaped loops corresponding to the rhombomeric segments.
J. HuC:GFP transgenic embryo at the same stage, imaged from the same aspect, showing the GFP-expressing neurons of the mantle zone are complementary to the GFAP-expressing glial curtains in Cii.
Scale bar: 100μm.

GFAP in the floorplate, and also punctate immunoreactivity throughout the white matter of the marginal zone (Figure 3.4, E). Staining by the classical periodic acid-Schiff reaction (McManus, 1946) reveals glycogen-rich processes in the same region as GFAP-expressing cells reside (Figure 3.4, F). These 'glycogen-rich' radial glia appear in the hindbrain between five and six days post-fertilisation, after the majority of neurogenesis has taken place and are in the same location as GFAP-expressing radial fibres (Figure 3.4, E).

GFAP and Markers of Neuronal Differentiation (acetylated tubulin and HuC)

HuC:GFP expression labels neurons in the mantle zone of the zebrafish CNS (Figure 3.2). Tg(HuC:GFP) specimens also labelled for GFAP show that these two markers demarcate the cell bodies of the mantle layer and ventricular zone respectively (Figure 3.5, A). There is very little overlap between GFAP-positive cell bodies and HuC:GFP-expressing cell bodies. GFAP-positive fibres project through the mantle zone to the pia and many HuC:GFP-positive neurons are found in close proximity to a GFAP-positive fibre bundle (Figure 3.5, A).

The relationship between GFAP-positive processes of the glial curtain and neuronal organisation was examined using the monoclonal anti-acetylated tubulin antibody (Table 3.1). This antibody was initially published as a marker of early axon tracts in the zebrafish (Wilson et al., 1990) but also labels many neuronal cell bodies. Acetylated tubulin immunoreactivity was found in the neuronal cell bodies of the mantle layer, in the longitudinal and circumferential axon tracts in the marginal zone and in radial bundles reminiscent of the glial curtain (Figure 3.5, B). Occasional immunoreactivity was observed in the ventricular zone, but generally this staining was weak and the VZ was found to be generally devoid of tubulin. When GFAP and tubulin were stained simultaneously, tubulin was expressed in some but not all of the strongly GFAP-positive basal processes of the glial curtain (Figure 3.5, B and b). The radial fibres generally expressed more GFAP than tubulin, although in a minority only one of the two antigens was expressed (Figure 3.5, b). In the marginal zone, longitudinal fascicles of tubulin-expressing axons are surrounded by the basal processes of the glial curtains that express both GFAP and tubulin (Figure 3.5, b).

Figure 3.5. Expression of glial fibrillary acidic protein (GFAP) with neuronal markers and monoclonal antibody zrf-1

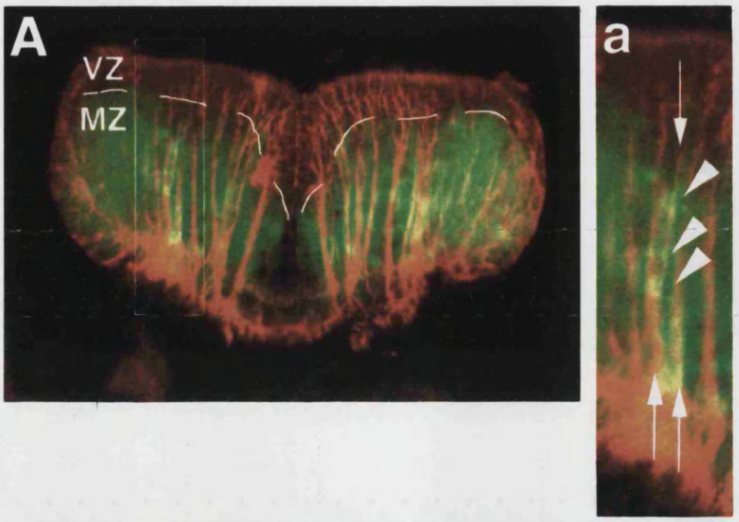
Wildtype or Tg(HuC:GFP) transgenic embryos immunostained and imaged in transverse section by LSCM. A and B are maximum intensity projections of z-series whilst C is a single confocal section. Panels a – c are higher magnifications of the same photomicrographs.

A. 42hpf. Cell bodies in a transverse section of the hindbrain are organised into two compartments, the GFAP-positive (red) ventricular zone (VZ) and the HuC:GFP-positive (green) mantle zone (MZ). The cells that are in close proximity to the strongly GFAP-positive bundles of cell processes in the MZ (arrows) are HuC:GFP-expressing neuronal cell bodies (arrowheads) (a, magnification of inset in A).

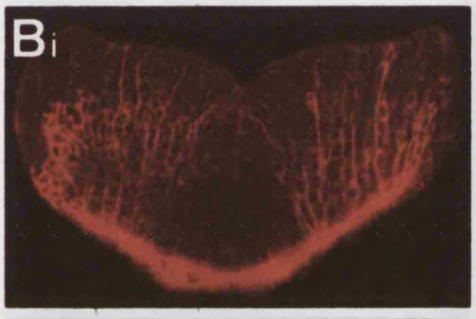
B. 48hpf. GFAP (green) with acetylated tubulin (red), labelling axons and some neuronal cell bodies. The lateral, tubulin-rich masses of cells bodies are hindbrain commissural neurons. The radial GFAP-positive fibres delineate and divide the longitudinal forming axon tracts in the white matter (arrows in b). Simultaneous demonstration of GFAP and acetylated tubulin reveals the heterogeneous nature of the glial curtains, with some fibres expressing only GFAP, others composed of both GFAP- and tubulin-positive processes and a minority, tubulin only (arrowheads in b).

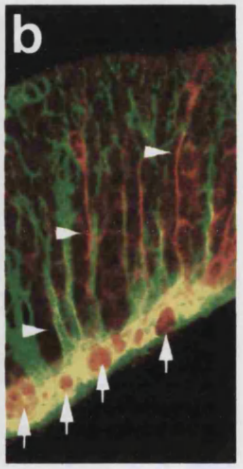
C. 48hpf. GFAP (green) and zrf-1 (red). Processes of the glial curtains staining positive for zrf-1 also express GFAP (double-staining processes in e). zrf-1 is restricted spatially to the basal extremes of the processes that make up the glial curtains.

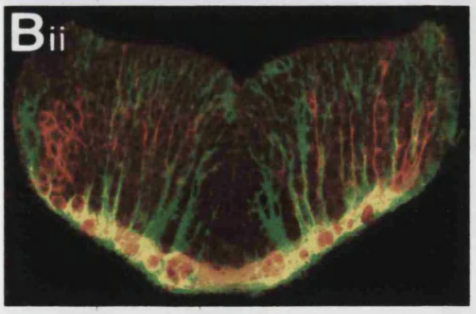
Scale bar: 100μm.

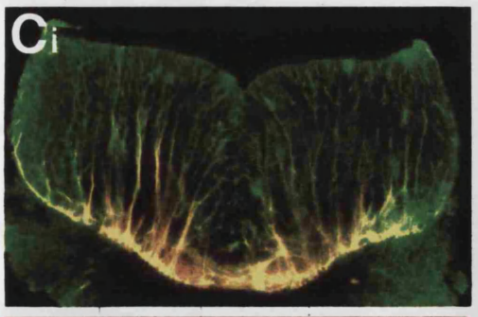

A
VZ
MZ

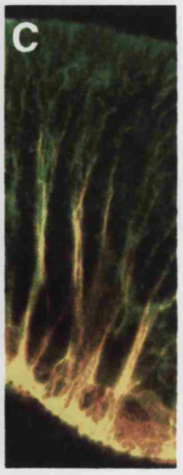
a

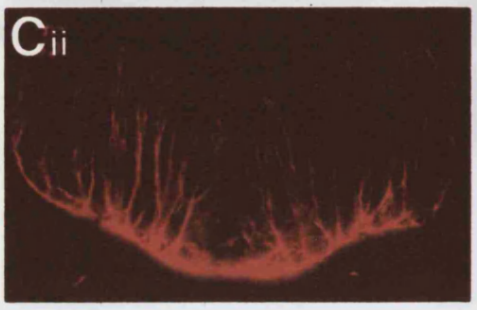

Bi


b


Bii


Ci


c


Cii

GFAP and zrf-1

The glial curtain was first visualised with a monoclonal antibody known as zrf-1 (Trevarrow et al, 1990). This antibody was raised to a zebrafish brain cytoskeletal extract of unknown composition (Trevarrow et al, 1990). Zrf-1 staining has features similar to both GFAP and acetylayed tubulin. Simultaneous immunostaining with GFAP reveals that zrf-1 is detected in the same radial processes that express GFAP but zrf-1 is more restricted to the basal regions of these processes (Figure 3.5, C). Zrf-1 immunoreactivity was not detected in the ventricular zone where GFAP-expressing cell bodies are prominent. In contrast to acetylated tubulin, zrf-1 staining is not found in cell bodies in the mantle layer but is found in the basal processes of the glial curtains, concentrated in the pial endfeet.

Other Markers of Radial Cells

In order to further characterise the GFAP-expressing radial progenitors in the VZ, a panel of mammalian antibodies was assembled for immunohistochemistry (Table 3.1). Markers reported in the literature to be markers of radial glia in mouse such as nestin, vimentin, RC2 and GLAST were used for wholemount immunohistochemistry. Vimentin, RC2, nestin (Rat-401) and GLAST were found not to react with wholemount zebrafish CNS. The S-100 antibody did not label any cells in the CNS either, but did label a glial subtype in the neuromasts of the lateral line. However, since these antibodies were raised to mammalian proteins for use in rodent model systems, the most likely explanation for their lack of reactivity in fish tissue is species specificity. On the other hand, some mammalian antibodies do cross-react well with fish tissue, including anti-GFAP, anti-tubulin and S-100, so there is clearly variability in the conservation of different proteins. But many mammalian antibodies that are specific to radial glia have not been useful in this study and zebrafish-specific antibodies are required for further characterisation.

GFAP and Markers of Proliferation

At 48hpf a minority of GFAP-expressing cells possess nuclei that express the phosphorylated histone-3 (PH3) antigen, indicating that these cells are in M-phase of the cell cycle (Figure 3.6, A). A single pulse of BrdU at 36hpf shows that an even greater

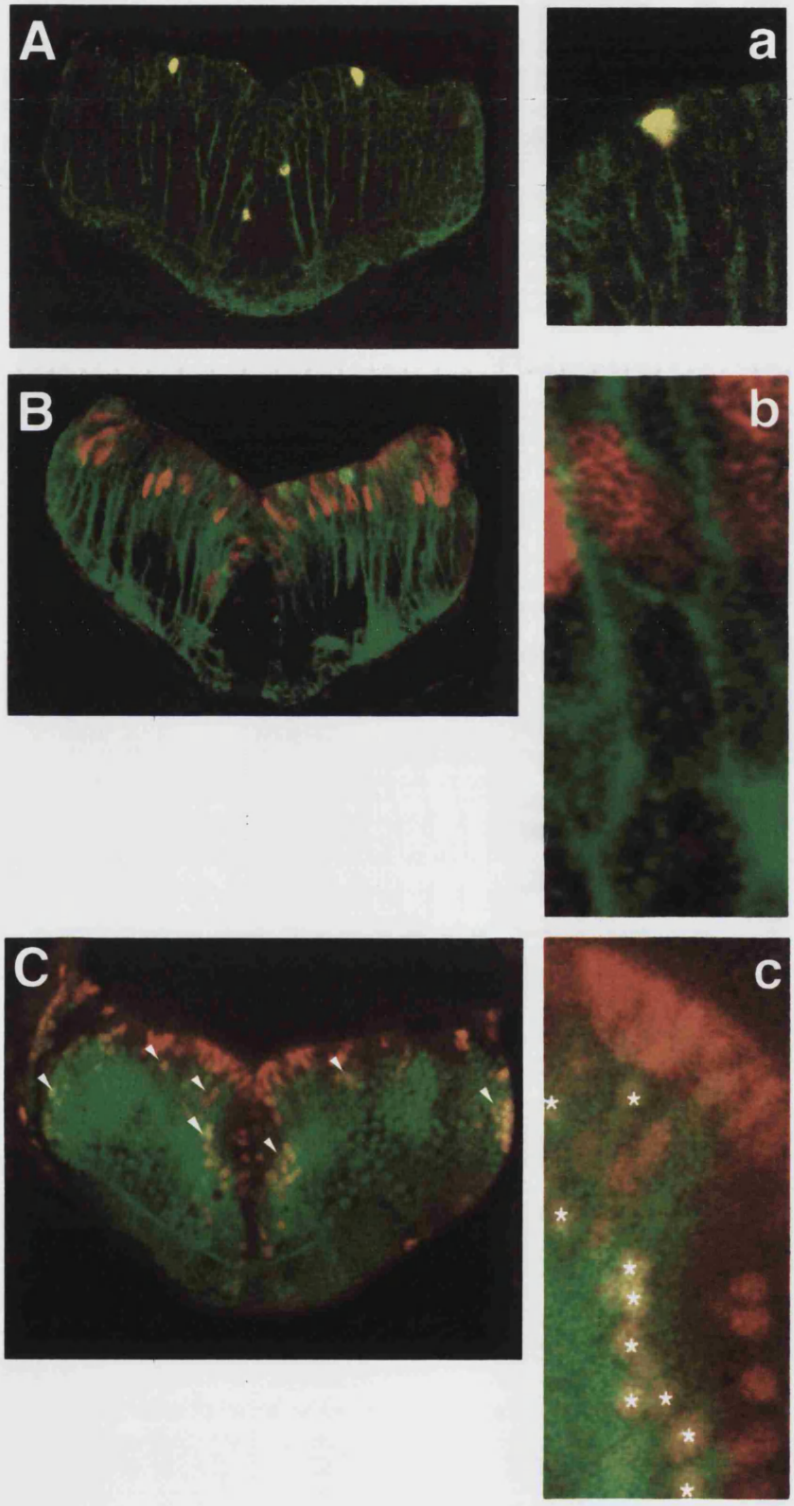
Figure 3.6. Expression of glial fibrillary acidic protein (GFAP) with markers of proliferation

Wildtype or Tg(HuC:GFP) transgenic embryos immunostained and imaged in transverse section by LSCM as maximum intensity projections of z-series. Panels a – c are higher magnifications of the same photomicrographs.

A. GFAP (green) with phosphorylated histone-3 (PH3, red). PH3 staining completely overlaps with strong GFAP immunoreactivity, generating yellow cell body profiles in the VZ (a).

B. Single acute pulse of BrdU at 36hpf reveals the plentiful cells in S-phase of the cell cycle; the position of cycling cells (red) delineates the VZ at this stage. Note how the MZ is devoid of BrdU-incorporating cells. The BrdU-positive nuclei are encircled by the GFAP-expressing cytoskeleton (b).

C. Single BrdU (red) pulse at 36hpf in the Tg(HuC:GFP) (green) transgenic followed by a 12-hour recovery reveals the GFAP-expressing, BrdU-incorporating VZ cells produce neurons (green)(arrowheads). The appearance of BrdU-positive, GFP-expressing neurons in the mantle zone 12 hours after pulsing demonstrates that BrdU-incorporating cells in the VZ are migrating into the MZ and differentiating into neurons (asterisks in c).

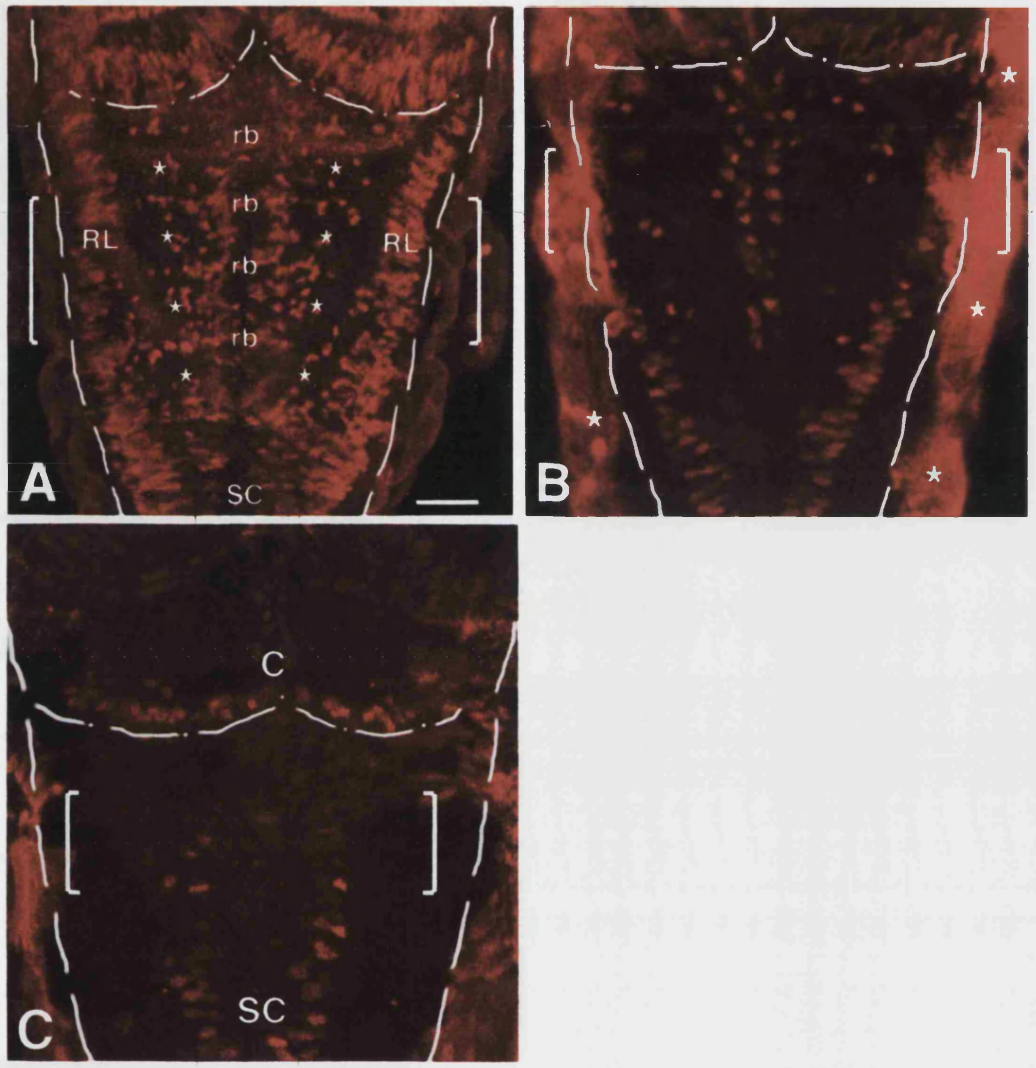

A
a
B
b
C
c

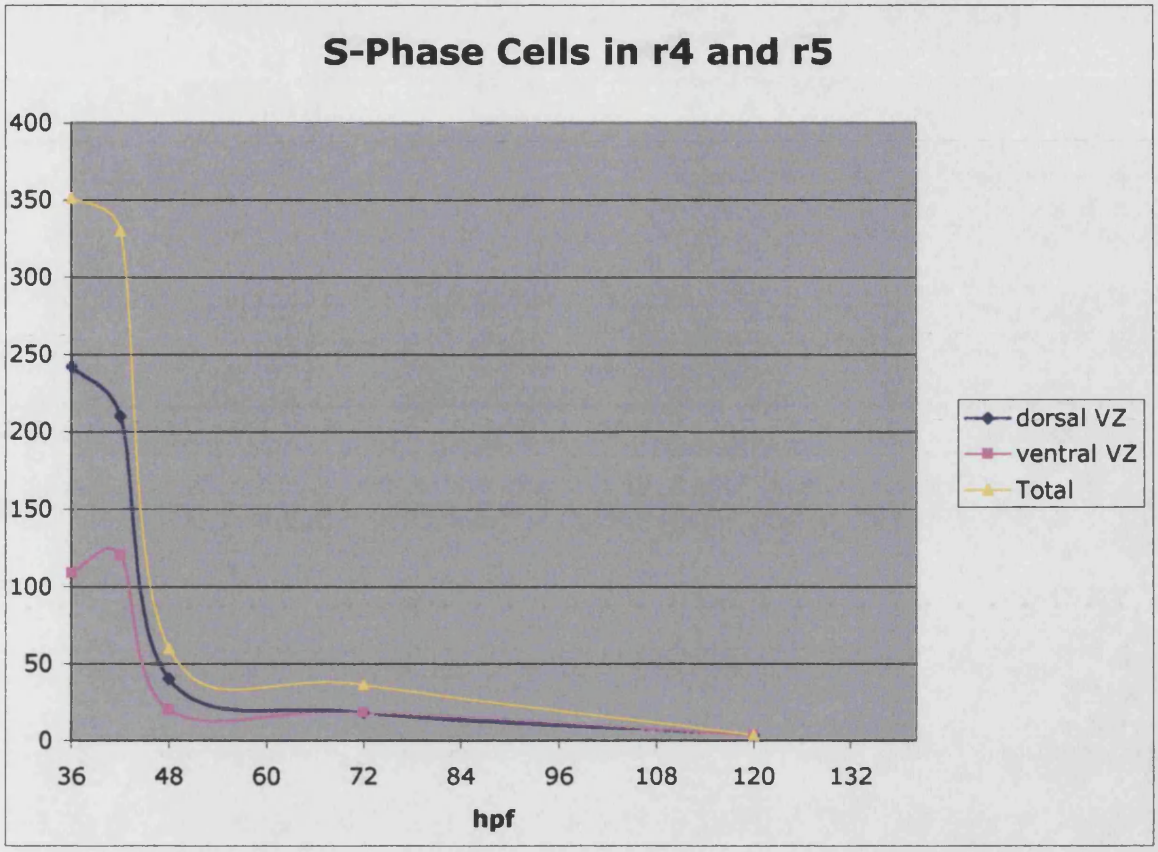
proportion of GFAP-expressing cells in the VZ are in S-Phase of the cell cycle, synthesising DNA (Figure 3.6, B), and a single pulse of BrdU applied to 36hpf Tg(HuC:GFP) transgenic embryos followed by a 12-hour recovery before detection of BrdU shows that these cells are generating neurons (Figure 3.6, C).

Analysis of Cell Proliferation in the Ventricular Zone

Previous studies (Lyons et al., 2003) have shown that hindbrain neurogenesis is mostly complete by hatching (72hpf), but my visualisation of the VZ using the Tg(HuC:GFP) transgenic during embryonic and early larval development has shown that there is clearly a non-neuronal, undifferentiated compartment of cells, that persists beyond hatching stage. To demonstrate the changes in proliferative capacity of the cells in the VZ with time, cells in S-phase of the cell cycle were visualised by pulsing wildtype embryos with bromo-deoxyuridine (BrdU) at single timepoints in development: at 36, 42, 48, 72, 96 and 120hpf (Figure 3.7). Labelled cells in rhombomeres 4 and 5 were counted by the Disector Principle (Sterio, 1984) and additionally scored as to their position in the VZ: as described earlier in this chapter (see Figures 3.2 and 3.5), the hindbrain VZ becomes divided into two discrete compartments, a ventral, medial group of cells and a dorsal layer of cells that form the floor of the 4th ventricle (see Figure 3.2). At 36hpf, a high proportion of cells occupying the VZ are in S-phase of the cell cycle, indicative of the high rate of neurogenesis that is taking place at this point of development (Figure 3.7, A). However, between 36 and 48hpf, the number of BrdU-incorporating cells suddenly drops by over 80% (Graph 3.1), and cells in cycle become progressively restricted to two discrete regions within the dorsal and ventral VZ (Figure 3.7, B). The former is comprised of the caudal rhombic lip that lies in the dorsolateral hindbrain VZ, whilst the latter is a medial domain positioned more ventrally. The medioventral domain lies just dorsal to the floorplate and is most likely the site of oligodendrogenesis later in development. However, beyond 48hpf the proportion of cells still remaining in S-phase declines in both domains. This correlates with previous studies that have shown in the zebrafish hindbrain, neurogenesis takes place at its highest rate at 36hpf, and then gradually declines (Lyons et al., 2003). However, even at 5dpf there are occasional cells in the hindbrain incorporating BrdU (Figure 3.7, C),

Figure 3.7. Quantification of cells in S-phase in rhombomeres 4 and 5
Embryos were pulsed with BrdU at single timepoints and allowed to recover for 30 minutes before sacrifice and detection of analogue incorporation by immunohistochemistry. All images are maximum intensity projections of confocal z-series imaged by LSCM from the dorsal aspect. Anterior is to the top. Broken line represents the lateral extremes of the CNS; broken line with dots delineates the cerebellar fold, and the square brackets represent the region of interest for counting (rhombomeres 4 and 5). BrdU-incorporating cell nuclei (red) in this region were quantified by the Disector Method (Sterio, 1984) using 3mm z-sections imaged through the D-V height of the embryo and counted manually in NIH Image.
A. 36hpf. BrdU incorporation is widespread throughout the hindbrain, in the centres of each rhombomere (asterisks), boundary regions (rb) and rhombic lip (RL). There is also strong incorporation of BrdU in the anterior spinal cord (SC), at bottom limit of the photomicrograph.
B. 72hpf. BrdU-incorporating cells become increasingly restricted to the rhombic lip and a prominent medial domain in the ventral hindbrain. The hindbrain becomes compressed in the A-P axis as the body axis straightens. The centres and boundary regions of the hindbrain are almost devoid of cells in cycle by this stage. This timepoint represents the end of embryonic development as the zebrafish larva hatches at this stage. The intense staining in the margins of the image is background fluorescence from secondary antibody binding to the skin (asterisks).
C. 5dpf. The cerebellum folds and flattens back over the neural tube and obscures much of the anterior hindbrain. There still remains a very small number (mean <5) of cycling cells in r4 and r5 by this stage. The spinal cord (SC) and the cerebellum (C) both display a low level of BrdU incorporation.
Scale bar: 100μm.


rb
rb
rb
rb
RL
RL
A
SC
B
C
C
SC



Graph 3.1. Quantification of cells in S-phase of the cell cycle in the hindbrain ventricular zone.
Embryos were pulsed with BrdU at single timepoints between 36 and 120hpf. Each timepoint is the number of cells incorporating BrdU within rhombomeres 4 and 5. BrdU-incorporating cells were scored as to their D-V position within the hindbrain: dorsal (blue) or ventral (magenta) ventricular zone (VZ). All specimens were counted by the Disector Method (Sterio, 1987) and each timepoint is the mean of three specimens.

although their number has declined to only approximately 5% of the number of BrdU-incorporating cells present at 36hpf.

Discussion

In this chapter I have identified and characterised the progenitors comprising the ventricular zone (VZ) in the developing zebrafish hindbrain. These progenitor cells possess a radial morphology, express the glial fibrillary acidic protein (GFAP) and their somata lie in the VZ, all hallmarks of radial glia as described by Rakic (Rakic, 1971a, 1971b and 1972). Additionally, I found many of these GFAP-expressing cells to be mitotically active, expressing the PH3 antigen. Analysis of BrdU-incorporation showed that some of these cycling, radial glia-like cells are neurogenic, corroborating recently published work in mouse that many radial glia are also neurogenic (Heins et al., 2000, Malatesta et al., 2000, Tamamaki et al., 2001, Hartfuss et al., 2001, Noctor et al., 2001, 2002 and 2004, Miyata et al., 2001, Malatesta et al., 2003 and Anthony et al., 2004).
By using the stable transgenic Tg(HuC:GFP) line I identified the VZ compartment in the hindbrain and show that in zebrafish, there are non-neuronal cells persisting in a VZ well into larval development, and some of these cells remain in the cell cycle. I have also identified a subpopulation of cells in the larval VZ that strongly express GFAP and extend radial, branched processes that terminate in endfeet at the pial surface. These cells appear approximately four to five days post fertilisation, long after the majority of hindbrain neurogenesis is complete and appear to be a mature, differentiated form of radial glia. I have therefore termed these cells 'mature' radial glia to distinguish them from the earlier-appearing GFAP-expressing cells.

GFAP Expression and Neurogenesis

Although Marcus and Easter were able to detect GFAP expression in the hindbrain as early as 15hpf (Marcus and Easter, 1995) I found that with my technique I could only detect immunoreactivity with confidence at approximately 24hpf. However, my data corroborate their finding in that the protein is mostly first localised to the pial endfeet of bipolar, neuroepithelial cells in the neural tube. Although they describe their numerous GFAP-expressing fibres and processes as glia, Marcus and Easter do not commit to

naming them 'radial glia' and do not try to identify the cells any further. Also, by their technique they were unable to identify the GFAP-expressing cell bodies of the hindbrain ventricular zone (VZ), nor did they perform simultaneous antibody staining with other markers. Here I performed immunofluorescence antibody staining to label GFAP in the embryonic hindbrain and was able to visualise not only the processes comprising the glial curtains but the cell bodies to whom these processes belonged. I have demonstrated here that the glial curtains described previously are composed of the basal processes of VZ progenitors, which fasciculate and condense into bundles as they project toward the ventral pia. A double immunofluorescent stain of GFAP with tubulin shows that there is an axonal contribution to the glial curtains too, explaining the previous observations that the glial curtains also stain for markers of neurons such as HNK-1 or tubulin (Marcus and Easter, 1995 and Lyons et al., 2003). Co-expression of tubulin and GFAP in the developing axons – especially noticeable in commissural axons – most likely demonstrates a phase very early in neuronal differentiation where the new-born neurons still express residual GFAP inherited from their GFAP-expressing VZ progenitor cell state as well as tubulin immunoreactivity characteristic of their new neuronal identity.

The expression of GFAP is biphasic during development of the zebrafish hindbrain. The first phase of expression correlates with embryonic neurogenesis: it begins to be expressed when neurogenesis is taking place, and is expressed by neuronal progenitors. According to Marcus and Easter, GFAP immunoreactivity is first detectable at 15hpf, which is the stage when the first neurons in the hindbrain are born (Marcus and Easter, 1995 and Lyons et al., 2003) and *in situ* hybridisation reveals RNA expression at this time too (Thisse et al., 2001 online database at http//zfin.org) although by my method I detected GFAP protein appearing slightly later. I show that GFAP immunoreactivity in the hindbrain peaks at 36hpf, which is also the time when the rate of hindbrain neurogenesis is at its peak (Lyons et al., 2003) and then GFAP expression declines as the rate of neurogenesis decreases past this stage. By 72hpf, when the majority of hindbrain neurogenesis is complete, GFAP is largely undetectable in this brain region. Therefore the first phase of expression of GFAP correlates very closely with the neurogenic period of embryonic development. In the macaque, Levitt and colleagues report that GFAP expression in the foetal cortex coincides with the period of development when neurogenesis takes place (Levitt et al., 1981). Unfortunately in rodents early VZ

progenitors do not express GFAP, so a comparison cannot be made. However, the recent study of Anthony and colleagues in the mouse demonstrated a similar strong correlation radial glial expression of brain lipid-binding protein (BLBP) with the timing of neurogenesis (Anthony et al., 2004).

The Glial Curtain

The glial curtain is a structure that appears to be unique to the zebrafish hindbrain. Therefore one must speculate as to its function, and why the zebrafish has evolved such a structure. Antibody staining to GFAP and tubulin shows that there is an overlap between the two antigens in the glial curtains (Figure 3.5, B). This implies there is a transition between ventricular zone, GFAP-expressing progenitor and mantle zone tubulin-expressing neuron. Also, all the progenitor cells of the hindbrain VZ, regardless of their A-P level, project their basal processes into the glial curtains. Therefore the glial curtains may exist to ensure that newborn neurons migrate in a specific fashion into the correct A-P position, and project their axons in an appropriate manner, since most cells are restricted from migrating in a rostrocaudal fashion by rhombomere boundaries (Fraser et al., 1990). In addition to the expression of GFAP in the glial curtains of the hindbrain, there is also a strong and persistent expression in the spinal cord at all A-P levels (see the online database of Thisse et al, 2001, at http://zfin.org). Therefore it may be possible that in both the hindbrain and the spinal cord, where neurogenesis takes place comparatively early in development, GFAP-positive radial processes and fibres may be providing a scaffold or physical guide to ensure neurogenesis is taking place in the correct manner. Unfortunately no GFAP mutant has been yet identified in the zebrafish and GFAP knockout experiments in mouse are inconclusive (see Chapter One). Gene knockdown in zebrafish using morpholinos could provide insight into the patterning role of GFAP-expressing cells.

There is no description in the literature of hindbrain glial curtains in other fish species. However, I did not test this by performing GFAP antibody staining in other fishes, for the duration of most of my project zebrafish was the only fish species kept at UCL. Now that both blind cavefish and medaka are available at UCL, a comparative survey among such diverse fish species, using GFAP and tubulin antibody staining, could be very interesting.

Are GFAP-Expressing VZ Cells Radial Glia?

It may be tempting to believe that the VZ progenitors in the embryonic hindbrain are radial glia due to their GFAP expression, timing of appearance and radial morphology. In my opinion however, that the cells express GFAP does not automatically make them radial glia, since expression of many markers including intermediate filaments and other cytoskeletal proteins is often transient, and very plastic in the embryo; for example, in some circumstances dividing progenitors have even been shown to express HuD protein or β-tubulin, (Memberg and Hall, 1995, Menezes et al., 1995 and Shen et al., 2002) which are usually used as neuronal markers, and nestin, a reported marker of stem cells and radial glia, has been shown to be expressed in some postmitotic neurons (Shen et al., 2002). However, it is also difficult to consider that these cells are neuroepithelial progenitors that happen to express GFAP by coincidence. GFAP is a marker of differentiated astrocytes, whilst neuroepithelial cells are considered the undifferentiated progenitor of the CNS, which makes this embryonic GFAP expression all the more enigmatic. Therefore, from immunohistochemistry and morphological characterisation alone it is not possible to be entirely certain of the nature of these cells, although so far the data from this chapter is highly indicative of radial glial phenotype. It was unfortunate that antibodies to RC2, nestin and GLAST, published markers of radial glia in mice, did not to react with wholemount zebrafish preparations. Further studies are required to investigate these radial glia-like progenitors, and this will be covered in the following chapters.

Identification of Larval GFAP-Expressing Mature Glia

I have described in this chapter that in the zebrafish hindbrain, the expression of GFAP is biphasic. In the second, larval phase of GFAP expression a very small group of radial glia-like cells becomes apparent at the dorsal midline from approximately 5dpf. These cells possess branched and complex processes that terminate in endfeet at the pial surface. In particular, on reaching the interface between the grey and white matter the branches become complex and spread extensively, and permeate the white matter. Staining paraffin-embedded 7dpf sections by the periodic acid-Schiff reaction reveals cellular processes rich in glycogen occupying the same D-V level and position as the

GFAP-expressing processes in antibody-labelled sections. I have termed these cells 'mature' radial glia to distinguish them from the earlier embryonic population. These cells may be a form of differentiated radial astrocyte, as they do not appear to incorporate BrdU, possess a complex morphology and express GFAP. Such astrocytic radial glia persisting well beyond embryonic development are widely reported in submammalian vertebrates such as amphibians, reptiles and fish (Stensaas and Stensaas, 1968a and b, Miller and Liuzzi, 1986, Holder et al., 1990, Bodega et al., 1993, Kálmán, 1998, and Abhoucha et al., 2004). This late-occurring, GFAP-expressing mature radial glia identified in this study is probably the zebrafish equivalent of these cells. It is speculated that in amphibians, these radial astrocytic cells are performing the functions that stellate astrocytes carry out in mammals (Miller and Liuzzi, 1986). Whether the same is happening in the zebrafish is not yet known, although in my study, GFAP antibody staining did not reveal any stellate or multipolar astrocyte in the hindbrain, up to 7dpf. Perinatal differentiation into stellate astrocytes is a known fate for mammalian radial glia (Choi and Lapham, 1978, Schmechel and Rakic, 1979, Choi, 1981, Voigt, 1989, Gaiano et al., 2000 and Schmid et al., 2003). Comparisons between teleost fish and mammals are difficult to make however, because stellate astrocytes have yet to be identified in the teleostan CNS outside the optic nerve (Nona et al., 1985 and Levine, 1989; for discussion see Kálmán, 1998) apart from the somewhat eccentric description of these cells in the spinal cord of the zebrafish (Kawai et al., 2001).

Closing Remarks

I have shown that during embryonic development, progenitor cells in the hindbrain VZ are expressing markers of radial glia (GFAP) and proliferation (Histone 3-PH3), and incorporating the DNA analogue BrdU; also, some of these BrdU-incorporating cells differentiate into neurons. To confirm and further examine the neurogenic potential of these proliferative, radial glia-like cells I fate-mapped them using a system of single cell labelling, and this is covered in the next chapter.

Chapter Four

Lineage Analysis of Hindbrain Progenitors During Late Neurogenesis

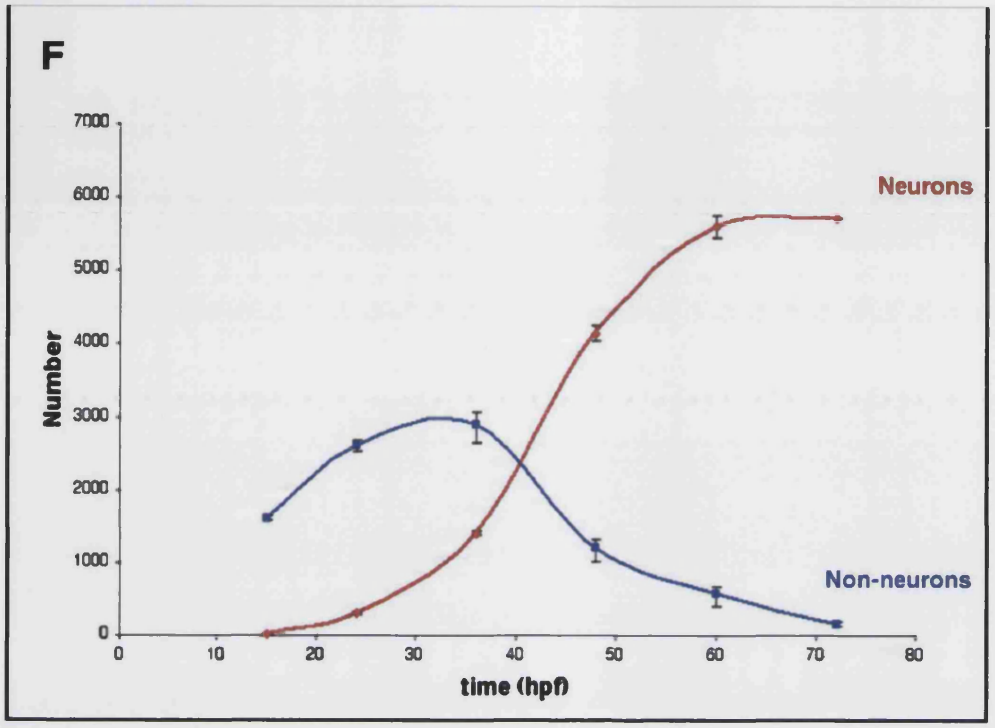
Introduction

Lyons and colleagues quantified zebrafish hindbrain neurogenesis from the first appearance of neurons in this brain region at approximately 15hpf until hatching, which takes place at 72hpf (Graph 4.1) (Lyons et al., 2003). The increase in neurons follows a classic sigmoid curve, whilst the progenitor cells increase transiently in number at around 36hpf before rapidly declining to approximately 500 cells per rhombomere by 48hpf, and to around 100 by 72hpf (Graph 4.1). My results in the previous chapter demonstrate the ventricular zone (VZ) cells that remain at 48hpf express the radial glial and astrocytic marker GFAP and that their basal processes are arranged in an unusual repeating pattern known as 'glial curtains' (see Chapter Three and Trevarrow et al., 1990, Marcus and Easter 1995 and Lyons et al., 2003). Despite being first described in the literature over a decade ago, very little is known about the significance of the glial curtain, whose arrangement appears to be unique to zebrafish. Antibody staining in the previous chapter demonstrated that the glial curtains contained both GFAP-expressing and tubulin-expressing fibres, implying they may be composed of both glial and neuronal components. My BrdU incorporation analysis also showed that at least some of these VZ cells are proliferative, and are also neurogenic. However, the precise fate and role of these late-occurring VZ progenitors is unknown.

The recent descriptions of neurogenic radial glia in the mouse forebrain have been an exciting development (Malatesta et al., 2000, Noctor et al., 2001, 2002 and 2004, Miyata et al., 2001 and Tamamaki et al, 2001 and Malatesta et al., 2003). Experiments in canaries (Alvarez-Buylla et al., 1990), lizards (Lopez-Garcia et al., 1988) and goldfish (Stevenson and Yoon, 1981) have long suggested that neurogenic radial glia also exist in submammalian vertebrates, in adulthood. In the rodent forebrain, the studies by Noctor and colleagues used timelapse video microscopy to show good evidence that radial glia were neurogenic via asymmetric, stem cell-like cell divisions (Noctor et al., 2004). However, this mode of neurogenesis may not be common to all regions of the CNS or in all vertebrates: Lyons and colleagues recently published an *in vivo* lineage study that

Graph 4.1. Population dynamics of neuronal and non-neuronal cells in the embryonic hindbrain

This graph, adapted from Lyons et al., 2003, shows the temporal increase in neuron number and the corresponding changes in non-neuronal cell number with development in the hindbrain (Lyons et al., 2003). These data are the result of counting the GFP-positive and GFP-negative cells in rhombomeres 4 and 5 in developing embryos of the Tg(HuC:GFP) transgenic line. The non-neuronal population is composed largely of ventricular zone (VZ) progenitors, although a small proportion will be newborn neurons that have yet to express HuC:GFP. The transient increase in progenitors early in development is characteristic of the transit amplifying model of neurogenesis. The rate of neurogenesis peaks between 36 and 48hpf, and levels off by approximately 60hpf. This chapter investigates the lineage and developmental fate of the progenitors found in the hindbrain VZ at 48hpf. At this stage the ratio of non-neurons to neurons is approximately 1:4 but this ratio decreases to 1:30 by 72hpf as the VZ is depleted of cells and more neurons differentiate.


F
7000
6000
5000
4000
3000
2000
1000
0
Number
0
10
20
30
40
50
60
70
80
time (hpf)
Neurons
Non-neurons

demonstrated in the zebrafish, 84% of hindbrain neurons born before 48hpf are derived from symmetric neuron-pair divisions (Lyons et al., 2003). In this chapter I will use single cell fate mapping techniques to examine the fate and mode of division of zebrafish hindbrain progenitors from 48hpf onwards. In particular, I aim to examine whether at these later stages the zebrafish progenitors continue to divide in a largely symmetric manner, or change their mode of cell division to an asymmetric one.

Methods & Materials

Single-cell labelling

48hpf wild type or Tg(HuC:GFP) (Park et al, 2000) embryos were dechorionated, anaesthetised in MS-222 Tricaine and immobilised in 1.5% low melting point agarose (Sigma) in embryo medium. To facilitate access to the brain, the skin over the region of interest of the embryo was treated with 10mgml-1 Pronase (protease from S. griseus, Sigma) for exactly two minutes before the dye labelling procedure. Microelectrodes were prepared from thin-walled 1.2mm OD aluminosilicate glass with internal filament (A-M Systems, Everett, WA) using a P-87 Flaming-Brown micropipette puller (Sutter, Novarto CA). The microelectrode was designed to be very sharp to ensure the chance of labelling a single cell and when filled, to have a resistance of approximately 150 megohms. When examined under 400x magnification with DIC optics the tip should not have a 'ribbed' or banded appearance but should come to a sharp, invisibly small tip with a blue-coloured taper. Microelectrodes were backfilled with a small amount of 5% 3,000 Mw biotin- and TRITC-conjugated dextran (Micro-RubyTM, Molecular Probes, OR) in sterile-filtered distilled water and then approximately 50ml 1M KCl immediately prior to use. I found that the Micro-Ruby™ was preferable to some of the other dextrans on offer in that it did not clog the microelectrode as easily even at high concentrations and had more favourable electrophoretic properties. Single cells in the hindbrain VZ were labelled by low amplitude current injection using a Neurolog amplifier (Digitimer). After advancing the microelectrode tip until it came to rest against the ventricular surface of the brain, a brief hyperpolarisation of approximately one second was sufficient to label a single cell. The current amplitude was between 2 – 8nA. Success of injection was monitored by epifluorescence microscopy. Following dye labelling, the

injected cell was examined under x400 magnification on a Zeiss Axioplan 2 microscope to ensure that no more than one cell was labelled and that the single cell was healthy. The embryo was immediately discounted from the study if the labelled cell was obviously extruded from the neuroepithelium or where more than one cell was labelled; generally if cell death occurred, it happened within minutes of dye injection, a timeframe reported in similar dye injection studies previously (Soula et al., 1999). When successful the single cell was photographed and the embryos incubated in embryo medium at 28oC for 24 hours. After this period the embryos were photographed again and the labelled clone phenotyped according to morphology alone or by morphology and GFP expression in the Tg(HuC:GFP) transgenic line. Overall, 50% of attempts at single-cell dye injection were successful, success measured by detectable and easily identifiable cells 24 hours post-injection.

Microscopy

Embryo sorting and staging was carried out on a Nikon SMZU dissecting microscope. Single cell labelling was performed on a Nikon Optiphot fixed-stage epifluorescence microscope with a 40x dry objective lens. The microscope was mounted on an air table to negate background vibration; this proved to be crucial when carrying out single cell injections. Immunostained, dye-injected or electroporated embryos were generally imaged by standard epifluorescence microscopy using a Zeiss Axioplan 2 microscope and 40x or 63x water immersion objective lenses. This microscope was linked to a Hamamatsu ORCA ER CCD camera and controlled using the programme 'Openlab' (Version 3.1.5, Improvision UK, Coventry). This software made possible automated z-series image capture and accumulation, volume deconvolution and timelapse microscopy. Single images from Openlab were transferred as Tagged-Image File Format (TIFF) files and imported into Adobe Photoshop (Version 6.0, Adobe Systems) for publishing purposes. A Leica laser scanning confocal microscope (Leica Microsystems, Heidelberg) was used to analyse certain fluorescent specimens such as those that could not be resolved by epifluorescence. Confocal scans and maximum intensity projections were captured on the confocal Leica microscope using Leica TCS NT software (Version 1.6.587) saved in Leica software format and then subsequently analysed by NIH Image

and Image J programs. Single confocal sections or projections were then imported into Adobe Photoshop for publishing purposes.

Long-term Lineage Tracing by Electroporation of Plasmid DNA encoding GFP

Electroporation was performed according to a technique based upon the protocol first described by Haas and colleagues (Haas et al., 2001) and modified in the Clarke lab by Marcel Tawk (see Concha et al., 2003). For both single cell labelling by intracellular dextran injection and electroporation a Nikon Optiphot fixed stage epifluorescence microscope with a 40x dry objective lens was used. The microscope was mounted on an air table to negate background vibration and stable micromanipulators with micrometre resolution were used to target cells. Wild type embryos at 48hpf were dechorionated, anaesthetised in MS-222 Tricaine and mounted in 1.5% low melting point agarose (Sigma) to hold the embryo in place inside a chamber device that facilitated embryo orientation and manipulation during the electroporation procedure. To facilitate access to the brain, the agarose covering the part of the embryo containing the posterior brain was removed and the skin in this region of interest was treated with 20mg.ml^{-1} Pronase (protease from *S. griseus,* Sigma) for exactly two minutes before the electroporation procedure. Microelectrodes were prepared from thin-walled 1.2mm OD aluminosilicate glass with internal filament (A-M Systems, Everett) using a P-87 Flaming-Brown micropipette puller (Sutter, Novarto). The microelectrode was designed to have a patch-like tip approximately three to five microns in diameter and a fairly steep shoulder, which was confirmed by x400 DIC light microscopy. Prior to the experiment the microelectrode was backfilled with 1-2ml of circular DNA (the plasmid was found to be expressed efficiently without linearization) encoding enhanced-GFP (EGFP) under the control of the a-tubulin promoter as described by Köster and Fraser (Köster and Fraser, 2000) and kindly provided by Steve Wilson. The DNA was suspended in sterile-filtered distilled water to a concentration of 1mg.ml^{-1}. Once filled with DNA the tip of the electroporation microelectrode was advanced towards the ventricular surface of the hindbrain. Once the tip was in contact with the surface of the brain, short pulses of voltage were applied across the embryo. In addition to varying the voltage, the number and duration of pulses was changed until a suitable schedule was determined. For 48hpf embryos, three 1-second trains of 200Hz pulses at 25V proved sufficient for

reproducible single-cell electroporation. Following electroporation the embryo was recovered from the agarose and allowed to develop normally in embryo medium for 7 days in a 28°C incubator. During this period the electroporated cell in the embryo was live-imaged either by epifluorescence or LSCM and then returned to the incubator and allowed to continue to develop. Since GFP was used as the intracellular tracer of these cells, the electroporation was carried out in wildtype embryos, and phenotyping was by cellular morphology.

Results

Single progenitors in the VZ of the hindbrain were labelled by intracellular injection of fluorescent tracer dye at 48hpf and examined 24 hours later. The resultant labelled progeny were phenotyped by morphology alone or by GFP expression in the Tg(HuC:GFP) transgenic line. In some cases it was possible to follow the cells for longer than 24 hours, although dilution of the dye and poor optical clarity of the older larvae reduced the chance of robust phenotyping and 72hpf was chosen as the endpoint for these studies. Additionally, since hatching takes place at 72hpf, this timepoint marks the end of embryonic development in the zebrafish.

Acute Observations of Single Hindbrain Progenitors

Acute observation of over 200 single cells injected with 3000 MW fluorescent dextran in the Tg(HuC:GFP) transgenic line reveals that GFP-negative progenitors of the hindbrain VZ possess a bipolar morphology similar to published reports of radial glia in other systems (Figure 4.1, B – F). The short apical process of each bipolar cell extended to the ventricular surface, i.e., the most dorsal extreme of the VZ, whilst the long basal-projecting process invariably reached the pial surface and therefore will be referred to henceforth as ventricular and pial process respectively. In injected cells, both pial and ventricular process was found to terminate in conspicuous rounded, or sometimes flattened, endfeet whilst the somata were generally slender, ovoid or spherical in shape (Figure 4.1, B – F). Due to their position however, some cells with their somata at the very surface of the VZ had the appearance of unipolar morphology, with some taking on a flattened shape or appearing even triangular by projecting cytoplasm along their basal

Figure 4.1. Single cell fluorescent dye injection in the hindbrain ventricular zone reveals cells with radial glia-like morphology

A - C. Single cell labelled with rhodamine dextran in a 48hpf Tg(HuC:GFP) transgenic embryo, immediately fixed and imaged in transverse section.

A. Green fluoresence superimposed upon DIC transmitted light. The ventricular zone (VZ) and mantle zone (MZ) are delineated by GFP expression.

B. Merge of green and red channels superimposed on transmitted light, revealing the dextran-injected single cell in the VZ.

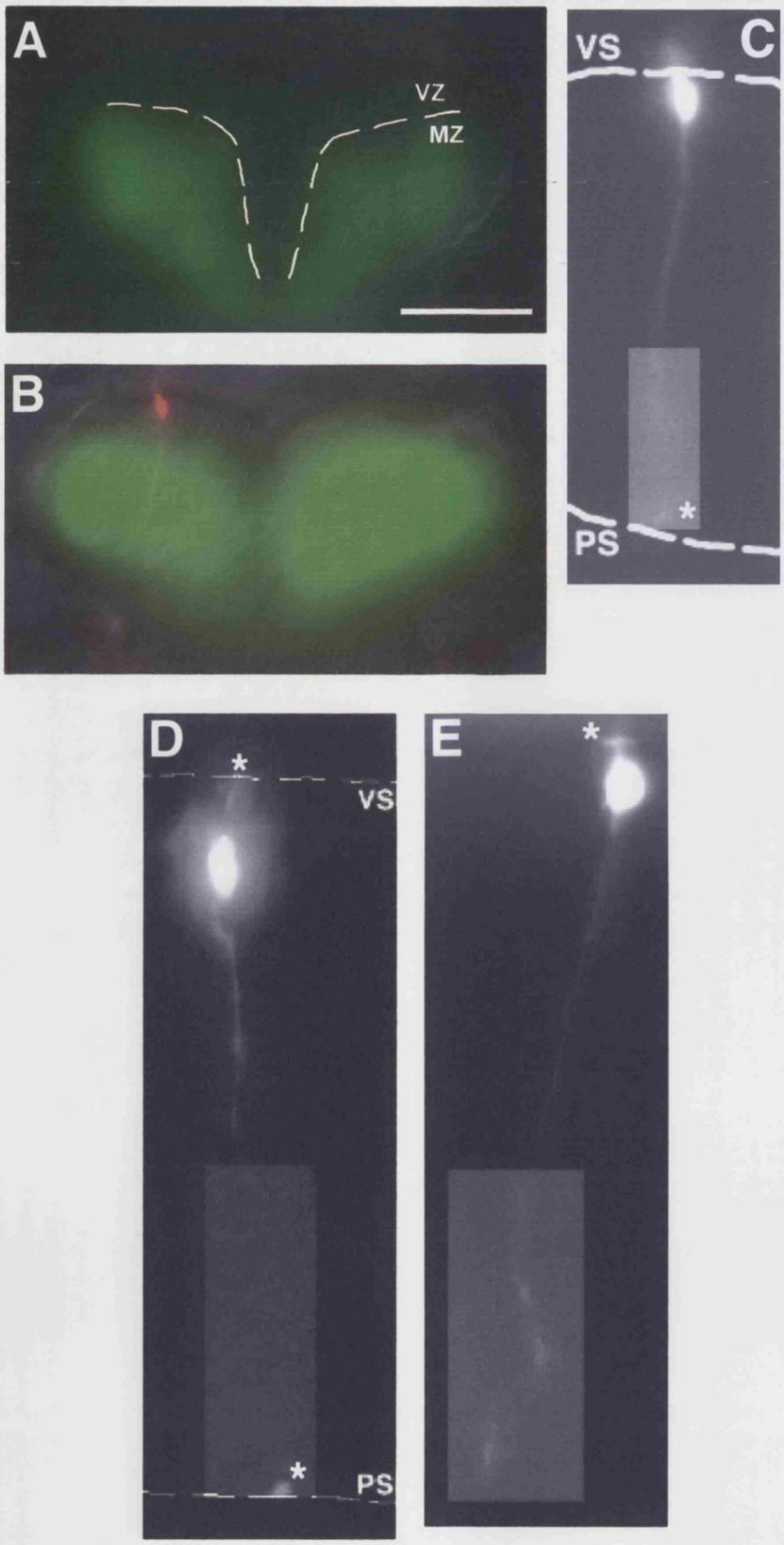
C. High magnification of the injected cell reveals bipolar morphology, with contrast-enhanced inset to highlight the pial process of the cell projecting to the pial surface and terminating in a widened endfoot (asterisk).

D – E. Dextran-injected single VZ cells displaying a variety of bipolar cell morphologies. Live imaged 48hpf embryos viewed from the lateral aspect. Ventricular surface (VS) and pial surface (PS) indicated in D by broken line, and insets contrast-enhanced to reveal endfeet.

D. Bipolar cell with fine processes extending to both VS and PS (asterisks). The pial-contacting process terminates in a rounded endfoot or club.

E. Bipolar cell with flattened, enlarged ventricular endfoot (asterisk)

Scale bar: 100μm.


A
VZ
MZ
B
C
VS
PS
D
VS
PS
E

process (Figure 4.2, A – C). Confocal microscopy was used to confirm that injected cells did not express GFP in the transgenic Tg(HuC:GFP) line (Figure 4.2, D).

24-Hour Fate Map by Dye Injection

Twenty-four hours after labelling cells were phenotyped and classified according to one of four behaviours: cell division, differentiation, somal translocation (radial migration) and quiescence.

Cell division

A small number of injected cells (6/101) were found to have undergone a single division. In every case observed, the two daughter cells lost contact with the ventricular surface and migrated away from the ventricular zone to lie the mantle zone (MZ). In the MZ the cells bodies took on a unipolar morphology with their single process directed towards the marginal zone (Figure 4.3, A – E). All of these observations strongly suggest both daughter cells differentiate into neurons. In one case, a cell division initially appeared to produce a single neuron and a radial, bipolar progenitor, but by 36 hours post-injection the bipolar cell had also lost its ventricular process, migrated into the MZ and took on the neuronal appearance of its sister cell (Figure 4.3, A and B). Without exception, the two cells in each of these radial clones were found to occupy a similar A-P level, with no cells observed to undergo a tangential, rostrocaudal or diffusive movement as observed in other systems (Leber and Sanes, 1995, Golden and Cepko, 1996 and Szele and Cepko, 1996).

Differentiation

In 34/101 cases the injected cell differentiated as a neuron without division. Cells were classed as having differentiated by fulfilling three criteria: their soma migrated into the MZ, they lost their short ventricular process and thus took on a monopolar morphology with their main process projecting into the marginal zone. When the dye injection was performed in Tg(HuC:GFP) transgenic embryos, the labelled cell was additionally confirmed as neuronal by co-expression of GFP (Figure 4.4, E). Although epifluorescence could indicate whether or not a cell soma lay in the mantle zone, to ensure the lineage-labelled cell also expressed GFP a small number (n=2) were imaged

Figure 4.2. Single cell fluorescent dye injection in the hindbrain ventricular zone reveals cells with radial glia-like morphology II

Live imaged 48hpf embryos viewed from the lateral aspect; A – C by epifluorescence, D by LSCM.

A - C Dextran-injected single VZ cells displaying a variety of cell morphologies.

A and B. Cells with their somata at the dorsal extreme of the neural tube possess a rounded or occasionally flattened shape, giving the appearance of unipolar morphology.

C. Cell with inverted triangle shape, due to its cytoplasm being drawn along its pial process.

D and D' Single dextran-injected cell in the Tg(HuC:GFP) transgenic demonstrates cells injected in this study are not neurons and lie within the ventricular zone. They are characteristically bipolar and radial in morphology.

D. Maximum intensity projection showing the radial glia-like morphology and pial process turning to avoid developing blood vessels (BV). The red-fluorescent cell body clearly lies outside the HuC:GFP domain.

D'. Red fluorescence only.

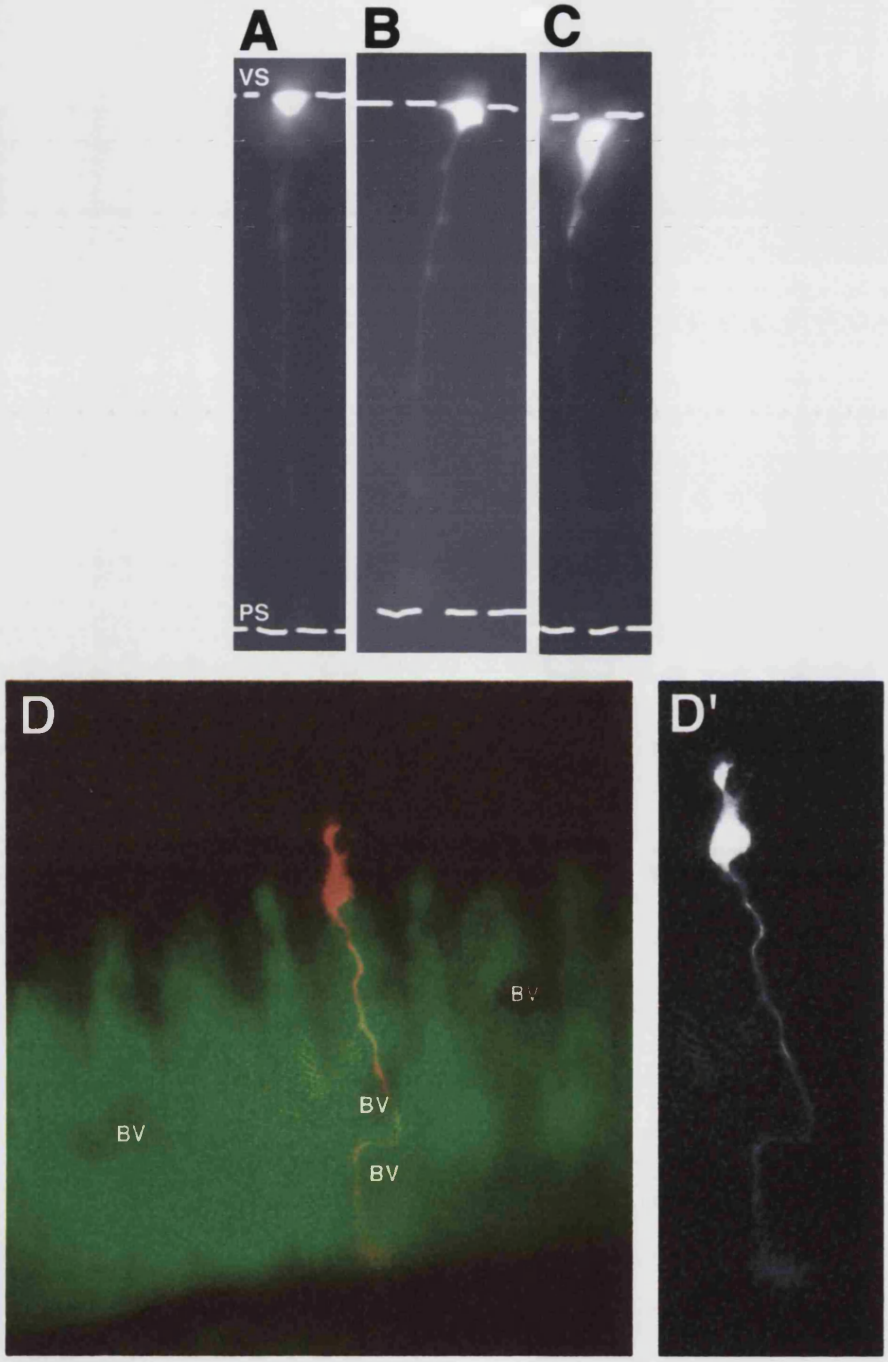

A
B
C
VS
PS
D
BV
BV
BV
BV
D'

Figure 4.3. Cell division

Cell division in the hindbrain VZ. Ventricular and pial surfaces are represented by broken lines at top and bottom of each image, respectively. Injected cells are all live-imaged from the lateral aspect, with anterior to the left.

A. At injection, the cell shows the characteristic morphology of VZ radial progenitors.

B. 24hpi. Cell division appears to have just taken place, with the two daughter cells adjacent. The left daughter cells has a monopolar, neuronal morphology whilst the cell tot he right retains a bipolar, progenitor-like appearance.

E. 36hpi. Despite the outward appearance of an asymmetric division at 24hpi, on examination twelve hours later both the daughters have migrated to a more ventral position, to within the mantle zone, and have taken on the appearance of differentiated neurons. The two daughter cells of this clone are positioned in a radial arrangement.

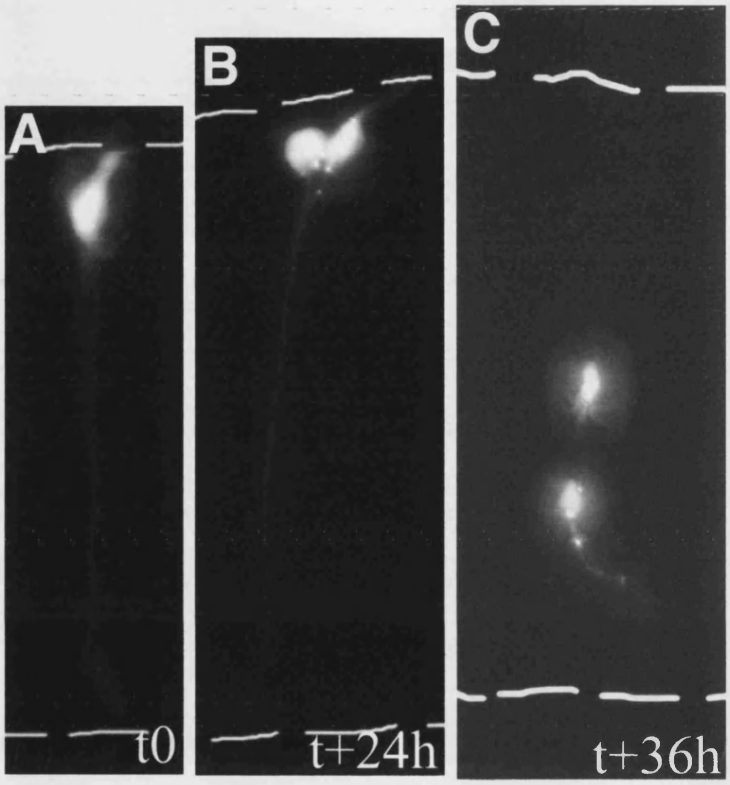

A
B
C
t0
t+24h
t+36h

Figure 4.4. Direct differentiation into neurons

Cells were phenotyped by either morphology alone (A, B) or by morphology and GFP expression in the Tg(HuC:GFP) transgenic (C - E). A - D are live imaged from the lateral aspect by epifluorescence, with anterior to the left, and ventricular and pial surfaces indicated by broken lines. E is a confocal section from a z-series imaged by LSCM from the lateral aspect at lower magnification; certain landmarks are labelled such as the cerebellar fold (CF), ventricular surface (VS), numerous blood vessels (BV) and the otic capsule (OC).

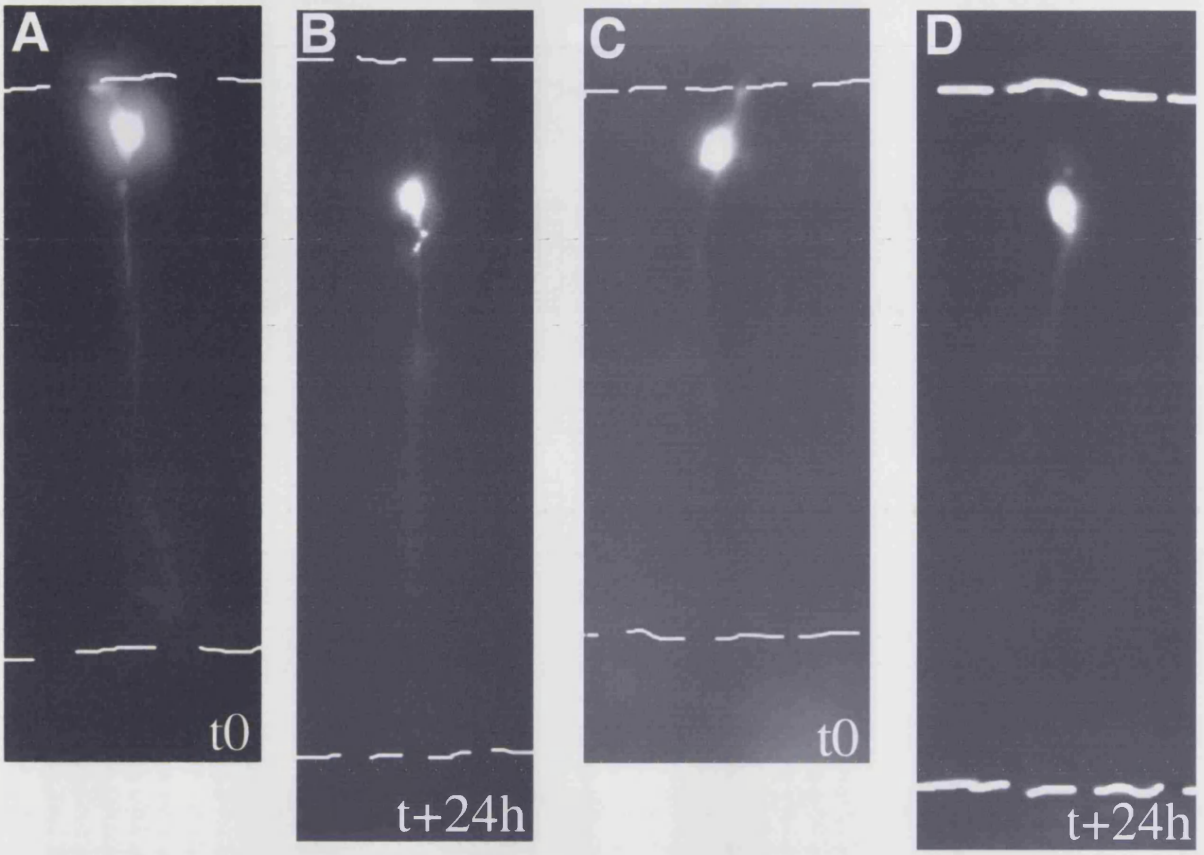
A. At injection (48hpf), the cell possesses typical bipolar morphology and cell body in the VZ.

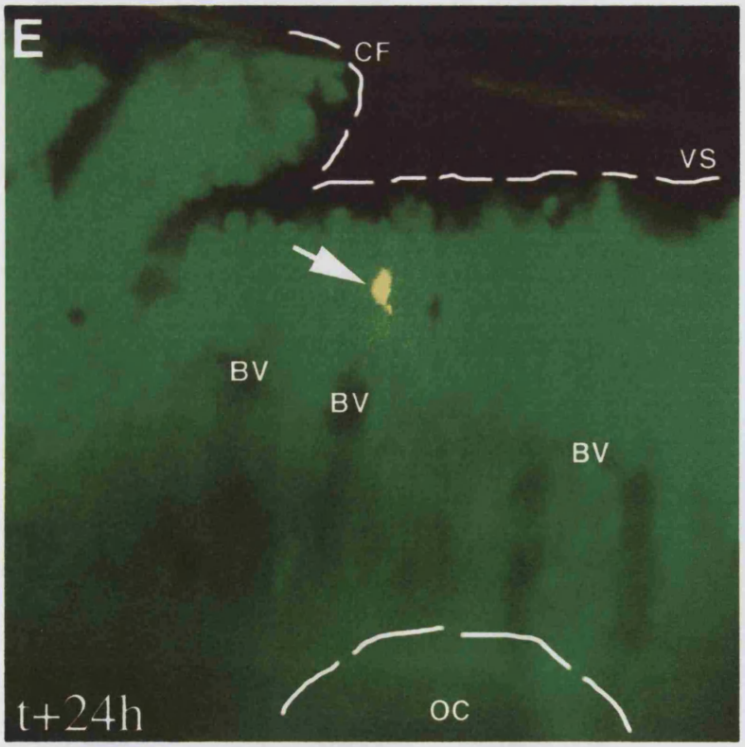
B. 24 hours post-injection (hpi) the cell has become monopolar, losing its ventricular process, and the soma is now located in a more ventral position in the mantle zone. Both imply a neuronal phenotype.

C. A separate specimen showing bipolar morphology at injection.

D. 24hpi the cell in C has lost contact with the ventricule, become monopolar and its cell body has moved into the mantle zone.

E. The specimen from D live-imaged by LSCM. A single confocal section shows the cell soma now in the HuC:GFP domain, further showing that this cell is a differentiated neuron.


A
t0
B
t+24h
C
t0
D
t+24h


E
CF
VS
BV
BV
BV
t+24h
OC

by confocal microscopy. Dextran-labelled cells well within the HuC:GFP domain were found to be expressing GFP and confirmed the reliability of my phenotyping. In no case did the long basal process of the VZ cell appear to be retracted before neuronal differentiation.

Somal Translocation from the VZ to the MZ

In 8/101 injected cells, the soma of the cell was found to migrate radially to a more ventral position, out of the ventricular zone and into the mantle zone, while maintaining its bipolar morphology and contact with the ventricular surface (Figure 4.5). In one instance it was possible to time this migration by timelapse microscopy, and the rate of radial migration was estimated to be approximately $10\mu m.hour^{-1}$. In these cases, the migration could be classified as somal translocation (Nadarajah et al., 2001). It is not possible to be sure of the eventual fate of these cells, however the migration of the cell body out of the VZ into the MZ suggests they may be in the first stages of neuronal differentiation, as a prelude to adopting a monopolar morphology.

Quiescence

In 53/101 cases the injected cell was found to exhibit none of the above behaviours. These cells retained their bipolar morphology with processes contacting both ventricular and pial surfaces, their cell bodies remained in the VZ and they did not express GFP in the Tg(HuC:GFP) line. These cells were defined as quiescent, for although their basal process became elongated as the hindbrain expanded in the D-V axis, the cells did not outwardly undergo any changes in behaviour (Figure 4.6). Again, to ensure these cells were not expressing GFP at a very low level undetectable by epifluorescent UV illumination, a small number (n=5) of labelled quiescent cells were imaged by LSCM

Figure 4.5. Radial migration by somal translocation

Radial migration by somal translocation. Ventricular and pial surfaces are represented by broken lines at top and bottom of each image, respectively. Injected cells are live-imaged from the lateral aspect, with anterior to the left.

A. Single progenitor cell at injection.

B. 24hpi, the cell body has clearly migrated to a more ventral position, whilst retaining its processes in contact with both ventricular and pial surfaces. The inset has been contrast-enhanced at the pial surface. This mode of migration has been described in the literature as somal translocation (see Nadarajah et al, 2001), although the maintenance of ventricular contact during this translocation was not reported in the rodent study.

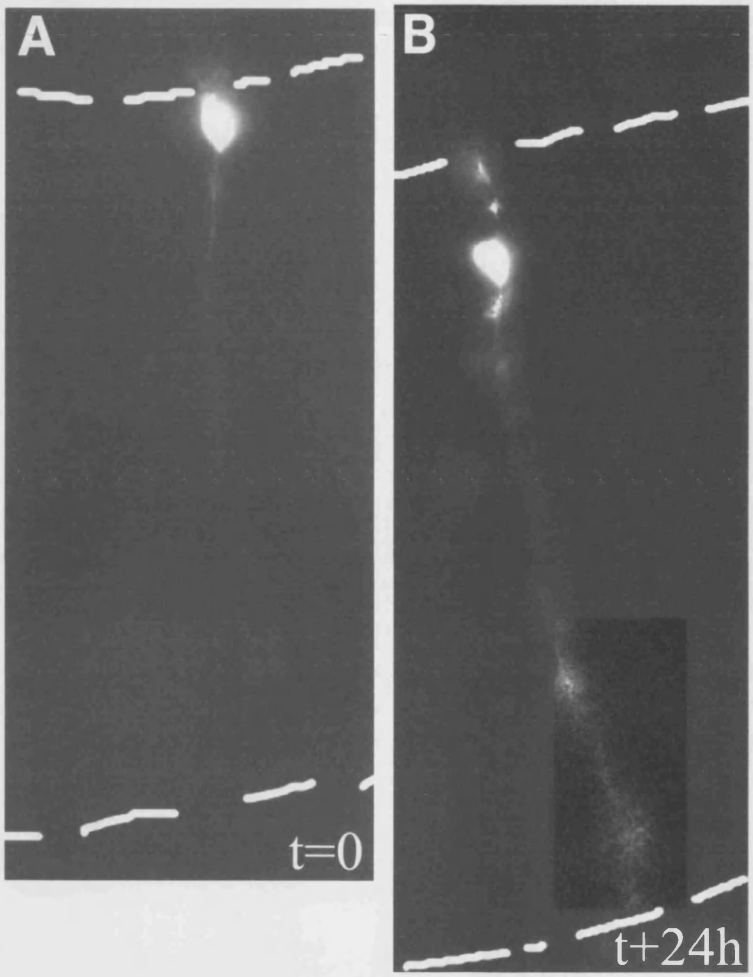
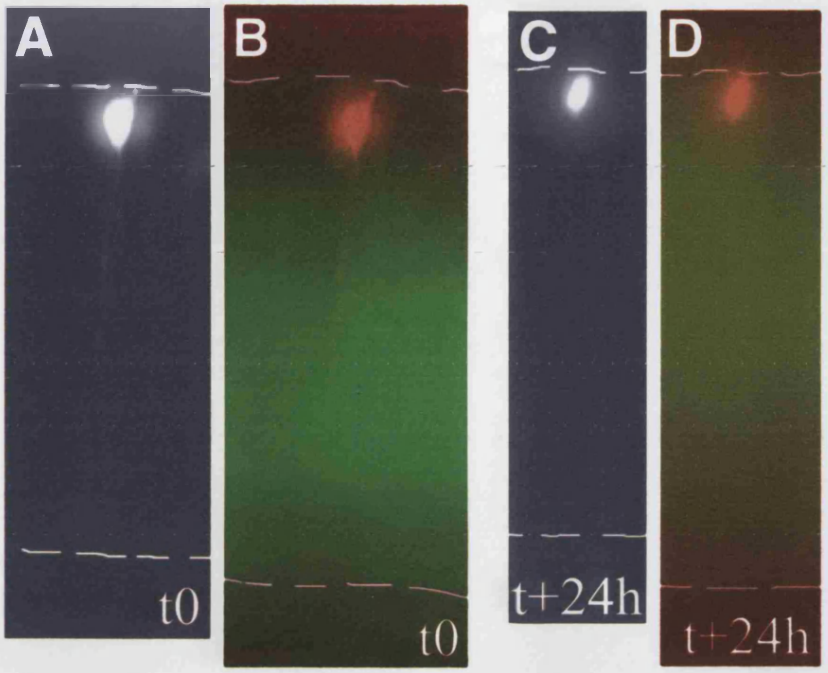
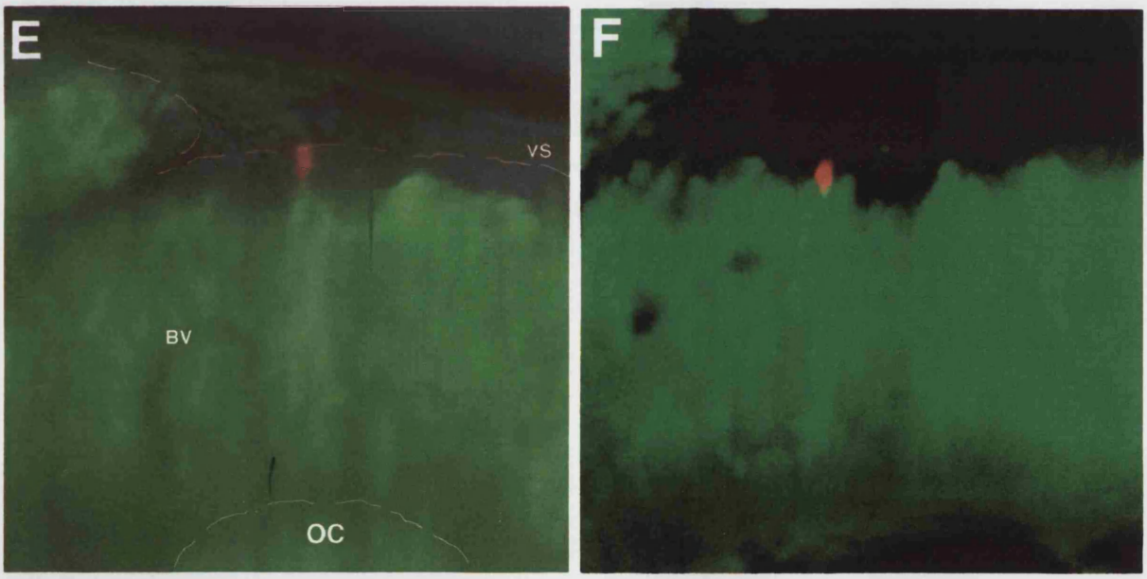

A
t=0
B
t+24h

Figure 4.6. Quiescent progenitors in the hindbrain ventricular zone
Injected cells which showed no change in D-V position, morphology or did not express GFP in the Tg(HuC:GFP) transgenic were classed as quiescent. A - D are live imaged by epifluorescence from the lateral aspect, with anterior to the left. E is a maximum intensity projection of a z-series onto DIC transmitted light with ventricular surface (VS), blood vessels (BV) and otic capsule (OC) labelled. F is a single confocal section from a z-series imaged by LSCM.
A. At injection the cell possesses bipolar morphology and it soma lies within the dorsal ventricular zone (VZ).
B. Image from A superimposed onto GFP fluorescence, showing the injected cell lies outside the HuC-GFP domain.
C. 24 hours post-injection (hpi) the cell soma does not change its D-V position and retains outside the HuC:GFP domain.
D. Image from C superimposed onto GFP fluorescence, showing the injected cell remains in the GFP-negative VZ.
E and F: Two quiescent cells live imaged by LSCM 24hpi to ensure that phenotyping by epifluorescence alone was a robust method.
E. Quiescent cell showing its position 24hpi relative to the VZ, the ventricular surface and the HuC:GFP domain. Green and red fluorescence projected onto DIC transmitted light.
F. Another specimen 24hpi showing the soma of a quiescent cell lying just dorsal to the HuC:GFP domain. Green fluorescence projected onto red.


A
B
C
D
t0
t0
t+24h
t+24h


E
VS
BV
OC
F

and both their lack of GFP expression, and the location of their cell bodies in the HuC:GFP-negative ventricular zone, was confirmed (Figure 4.6, E and F).

Summary of Dextran Injections

By the above criteria, the developmental fate of 101 progenitors in the hindbrain VZ was followed by intracellular injection of fluorescent dextran (Table 4.1). Of these, 6% underwent a single cell division to give rise to two differentiated neurons. A further 8% were observed to undergo a radial migration, or somal translocation, of their cell body whilst 34% directly differentiated into neurons without undergoing a cell division. The majority of progenitors observed in his study however, 52%, were quiescent and did not divide, differentiate or change the D-V position of their soma throughout the 24 hour observation period.

Long-Term Fatemapping Reveals Low-Level Neurogenesis Persists During Larval Development

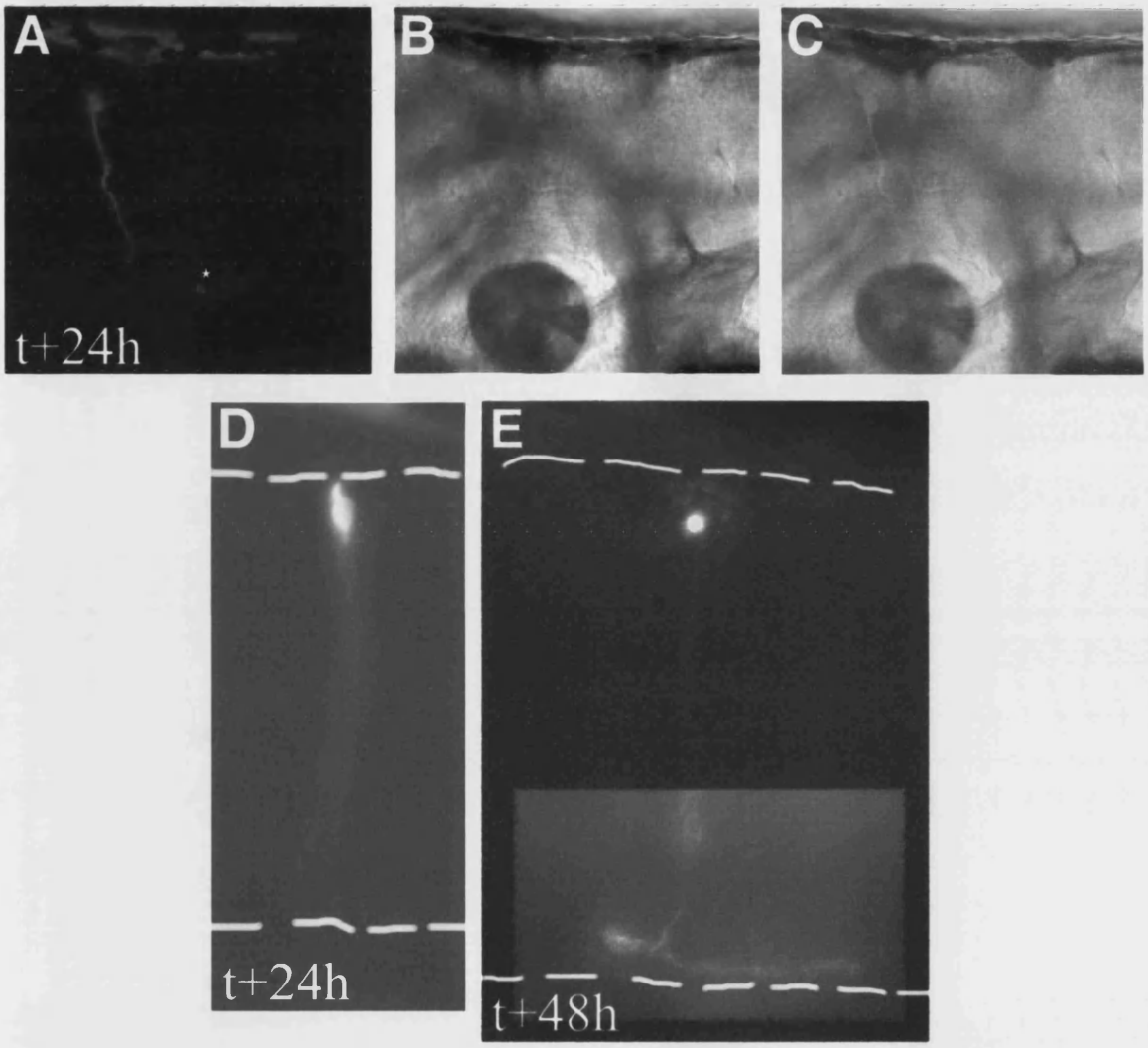
The use of single cell dextran injection only allowed cells to be followed with ease for 24 hours; beyond this time the increased tissue thickness and dilution of fluorescent dye hindered accurate observation of the injected cells. In order to increase the observation time of VZ cells up to one week the technique of single-cell electroporation has been employed. In this technique a plasmid encoding EGFP under the control of either the atubulin or EF1 promoter is electroporated into single cells via a patch-type pipette. The resulting expression of EGFP is persistent, very bright and fills all the processes of the labelled cells. In this way the developmental fate of progenitors in the hindbrain VZ was followed from 48hpf to 9 days post-fertilisation (dpf). One of the disadvantages of electroporating plasmid into cells is that until the EGFP DNA has been transcribed and translated, and the protein matured, the electroporated cell remains invisible. Cells can thus only be characterised no sooner than approximately 12 hours post-electroporation.

Twelve successful electroporations were carried out in this study. Success was measured by GFP expression in a single, healthy cell 24 hours post-electroporation (hpe). The cell was then monitored over the next six days, to observe changes in its behaviour or morphology. At the first visualisation, 9 of these cells had differentiated into a single neuron (Figure 4.7, A – C); this phenotype was confirmed by a number of

Behaviour	n	%
Quiescent No change in D-V position or processes	53	52.5
Neuronal Differentiation Loss of contact with ventricular surface/express GFP in HuC-GFP line	34	33.6
Somal Translocation Radial migration of soma whilst retaining bipolar morphology	8	7.9
Cell Division Cell undergoes one symmetric division producing 2 neurons	6	5.9
Total	101	100

Table 4.1. Summary of lineage study by single cell dye injection

Figure 4.7. Lineage analysis by single-cell electroporation
GFP-expressing specimens were live imaged 24 hours post-electroporation (hpe) to ascertain if single cell expression of GFP was achieved. Images are maximum intensity projections of z-series. A – C, by LSCM and D and E, by epifluorescence. In D and E, ventricular and pial surfaces are indicated by broken lines at top and bottom of the image respectively.
A. GFP-expressing single neuron with monopolar morphology, axon and growth cone (asterisk) imaged from the lateral aspect. Anterior is to the left. Nine out of twelve specimens showed such neuronal phenotype 24hpe.
B. DIC-transmitted light of specimen from A.
C. Green fluorescence projected onto transmitted light of specimen A. The proximal axon is partly obscured by one of the otoliths in the otic capsule.
D. By 24hpe some cells display a bipolar morphology reminiscent of the 'quiescent' cells identified in my dye injection study. This cell has a prominent process in contact with the ventricular surface.
E. 48hpe the cell in D has adopted a monopolar, neuronal morphology. The area at the bottom of the image has been contrast-enhanced to reveal the developing axon and growth cone.


A
t+24h
B
C
D
t+24h
E
t+48h

criteria. Their soma was located in the mantle zone and showed a rounded or polygonal morphology with a single process extending toward the pia. On close examination they possessed sufficient fluorescent signal to follow their projections into the axon tracts and locate their growth cones, thus unequivocally establishing their phenotype as neurons (Figure 4.7, A – C). Occasionally, after approximately 5dpe, some cells showed the first signs of dendritic elaborations forming on their soma, providing further convincing evidence for their neuronal phenotype (Figure 4.8).

At first visualisation the remaining thre of the GFP-expressing single cells retained a radial, progenitor-like phenotype, possessing all the morphological characteristics of the quiescent progenitors of my intracellular dextran injection study (Figure 4.7, D and E). When observed again at 48hpe, all three cells had attained the neuronal phenotype as described in the previous paragraph. Therefore between 72 and 96hpf, these cells had undergone a differentiation event (Figure 4.7, D and E).

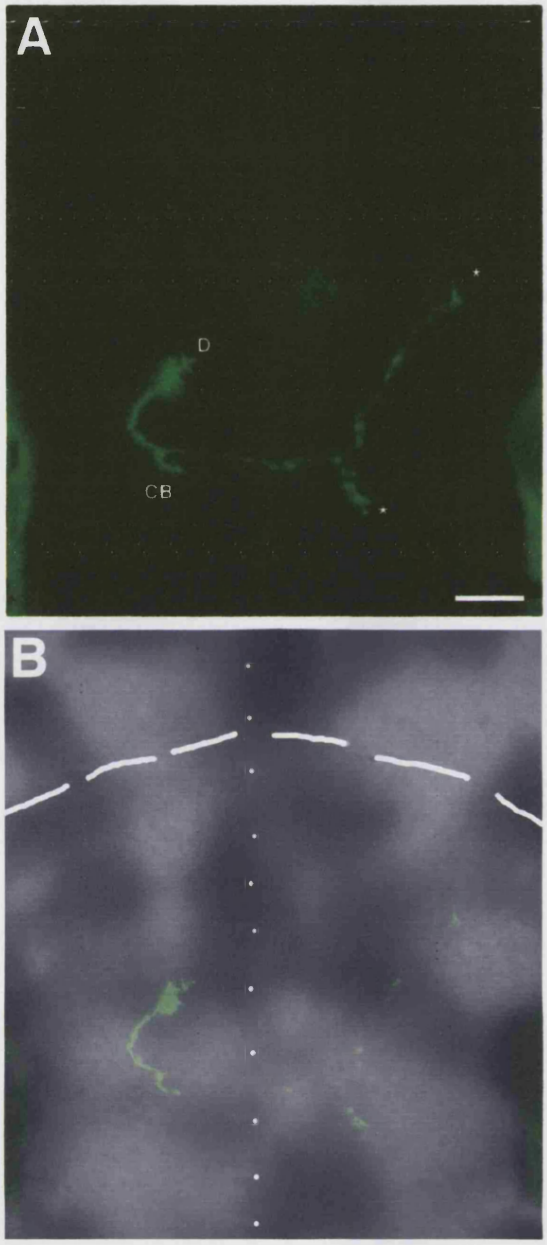
In 3/12 cases it was possible to further classify the neurons by their axonal projections as commissural interneurons (Figure 4.8). In other cases, it was not clear into what subtype of neuron the electroporated cell had differentiated, by morphology alone.

The aim of this experiment was to perform a long-term fate map of the progenitor cells in the hindbrain VZ at 48hpf. My dye injection study showed that up to 50% of these cells did not divide, differentiate or migrate during the 24-hour period leading to larval hatching. In this experiment, where fate was recorded for one week of larval development, all 12 of the VZ progenitor cells electroporated at 48hpf were found to be ultimately fated to give rise to neurons. In each case they did this by direct differentiation into a neuron rather than by cell division.

Discussion

In this Chapter I have mapped the developmental fate of hindbrain VZ cells – identified as GFAP-expressing progenitors in the previous chapter – using a combination of in vivo single-cell dye injection and single-cell electroporation. I have shown that during the latter stages of embryonic hindbrain neurogenesis, cell division is a relatively rare occurrence (<10% of clones) and those divisions observed were without exception, neuron-pair terminal divisions. None was asymmetric. However, most VZ progenitors

Figure 4.8. Long-term fate mapping by single-cell electroporation
GFP-expressing specimens were imaged 7 days post-electroporation (dpe). Embryos were live imaged by LSCM from the dorsal aspect. Images are maximum intensity projections of z-series. Broken line represents the midbrain-hindbrain boundary; dotted line the midline.
A. GFP-expressing single neuron with contralateral axonal projection terminating within the hindbrain. The soma possesses elaborations that may be developing dendrites (D). Its axon crosses the ventral midline and then bifurcates on reaching the contralateral side of the brain. One branch is visible as a short caudal projection, whilst the other is longer and extends rostrally. Both projections show numerous varicosities along their length and are tipped with growth cones (asterisks). The axon also displays a short, ipsilateral collateral branch sprouting in the caudal direction (CB). These projections are characteristic of commissural interneurons.
B. Image from A projected onto transmitted light.
Scale bar: 50μm.


A
D
CB
B

directly differentiate into neurons without undergoing mitosis. As many as 50% of VZ cells at 48hpf remain quiescent for at least 24 hours before differentiating into neurons. However, long-term fate mapping suggests that most of these cells also differentiate into neurons by 7dpf. Therefore the cells I classed as 'quiescent' may be progenitors differentiating into neurons very slowly, rather than cells entering a phase of behavioural arrest. Differentiation or transformation into stellate astrocytes, mature radial glia or oligodendrocytes was not recorded.

GFAP-expressing radial progenitors make neurons

The results presented in this and the previous chapter demonstrate that the large majority of GFAP-expressing radial progenitors in the zebrafish hindbrain give rise to neurons. Although previous work in rodent brains has been able to follow the fate of whole populations of radial neurogenic progenitors in vivo (Malatesta et al., 2003, and Anthony et al., 2004) this is the first study that has been able to look in detail at the behaviour of individual cells in an intact vertebrate brain during this process. In general my study is in good agreement with the neurogenic role described for the radial progenitors in mouse, however, the mode of neuronal generation is a little different. Firstly, none of the radial cells in my study has been seen to undergo self-renewing, stem cell-like asymmetric divisions that generate a progenitor and a neuron as daughter cells, as described in rodent brain slice cultures (Tamamaki et al., 2001, Miyata et al., 2001 and Noctor et al., 2001, 2002 and 2004). Only a very small minority (6%) of the progenitors in the 48hpf fish hindbrain undergo mitosis and in each case observed both daughter cells differentiate into neurons. In fact, from 48hpf onwards the majority of GFAP-expressing radial progenitors differentiate directly into neurons without division. Thus in both the early stages (Lyons et al., 2003) and late stages of neurogenesis, as shown here, no progenitors have been found that undergo multiple rounds of stem cell-like asymmetric division in the zebrafish hindbrain. The reasons for this major difference between rodent cortex and teleost hindbrain are not clear but may be linked to two things. Firstly, the fish embryo needs to generate its required neurons in a very rapid manner in order to have a functioning hindbrain by 72hpf. The quickest way to achieve this is via multiplicative symmetric divisions to rapidly expand the progenitor population, and then for these cells to undergo symmetric, terminal neuron-pair divisions

to generate as many neurons as possible in a final round of division (see Lyons et al., 2003). Secondly, compared to the fish hindbrain the mammalian cortex requires a huge number of neurons for functionality, yet embryonic neurogenesis in mammals occurs over a longer period of time. Therefore the very large numbers of neurons required can be generated by asymmetric, progenitor-neuron divisions at a slower rate, over a longer timeframe, whilst maintaining a constant progenitor stock (see reviews by Wodarz and Huttner, 2003 and Roegiers and Jan, 2004).

The Mechanics of Neuronal Differentiation: Transition from Progenitor Morphology to Neuronal

The exact sequence of morphological differentiation from a progenitor to a neuron is not precisely known. Again, studies in mammals are limited by the difficulty of making direct observation of this process taking place. Although the main aim of this chapter was not to record the dynamics of differentiation events, using the observations made here and in the previous chapter it is possible to speculate on the sequence of events that takes place during neurogenesis in the zebrafish hindbrain.

When a progenitor transforms into a neuron, there are many aspects of this transition that must be considered. First, neuroepithelial cells have a bipolar, radial phenotype, with processes spanning the full width of the neural tube and contacting both ventricular and pial surfaces; what happens to these processes as the newborn neuron develops appropriate axonal projection and dendritic connections is not known. A number of fates could befall these processes. For example, both the ventricular and pial processes could be retracted by the cell, and a new axon grown from the cell soma, or the pial process recycled into a proximal axon, which then develops a growth cone and grows in the direction appropriate for its neuronal type. From studies in mouse cortical slice culture, newborn neurons appear to partly retract both apical and basal processes to give the cells short leading and trailing processes, and with this morphology they migrate to the cortical plate (Nadarajah et al., 2001). In his detailed survey of neurogenesis in opossum forebrain using rapid Golgi staining, Morest describes many cell types and transitional forms in the developing forebrain, noting that "some of the young neurons have already formed their axons and early dendritic branches but still retain connections with the internal and external limiting layers" (Morest, 1970). This was especially

noticeable in pyramidal neurons of the anterior cingulate area of the forebrain. Morest also reports that it appeared these processes were later lost, by apparently being withdrawn or retracted by the soma rather than by simply degenerating or collapsing. Therefore in the opossum, newborn neurons were retaining the bipolar morphology of their immediate predecessors whilst developing their axons and dendritic connections separately. In amphibians, Holder and colleagues report differentiated motor neurons in the spinal cord sometimes retaining a fine process in contact with the central canal (Holder et al., 1991) that may be the remnant of an apical, ventricular process.

It is clear from the present study that in the zebrafish, a different sequence of events is taking place. Differentiating neurons retain the basal process of their immediate progenitor and transform this process into the axonal projection. This transformation probably explains why some axons retain GFAP expression (this is especially marked in tangential commissural axons: see Figure 3.6 in the previous chapter) and why some of the radial fibres that comprise the glial curtains express both GFAP and acetylated tubulin. These radial processes thus express markers characteristic of both their precursor cell type, and their newly differentiating state as a neuron. The exact mechanism by which the basal process of a recently differentiated cell transforms into an axon is also not clear. However, acute observations made of injected single cells in this study showed that all VZ cells invariably maintain contact with the pial surface by their basal process, with cells of a possibly transitional nature, i.e., withdrawing their basal process, never observed. It is not yet known at what stage of differentiation it loses its expanded contact with the pial surface and transforms into a growth cone, but these observations suggest that in the absence of progenitor division release of the basal process from the pial surface is a relatively late event in the progenitor-to-neuron transition.

The Mechanics of Neuronal Differentiation: Migration Out of the VZ

In addition to the transformation from progenitor cell morphology to neuronal, the newborn neuron must also migrate to a position appropriate for its cell type i.e., out of the VZ and into the mantle zone (MZ). It was not possible to completely determine the morphology of single newborn neurons as they migrated from the VZ and into the MZ, but there is suggestive evidence from the single cell dextran injections in this study. A

small number of cells were observed to undergo somal translocation into the mantle zone without release of their ventricular contact. If these cells are in the first steps of neuronal differentiation then this suggests release of the ventricular process is not necessary for this process; this contrasts with what has been described in the mouse cortex, where somal translocating cells lose contact with the ventricle as they migrate radially out of the VZ (Nadarajah et al., 2001). Given the rapidity of radial migration observed and the smaller proportion of bipolar, somal translocating cells (8%) compared to monopolar, differentiated neurons (34%) observed in my dye injection study, then the likely sequence of events is a rapid somal translocation from VZ to MZ whilst retaining bipolarity, followed by loss of the ventricular surface contact and adoption of monopolar morphology. No cell was found to regrow a contact with ventricle following this attainment of monopolar morphology, nor were cell bodies in the MZ with a trailing process observed, as described by both Nadarajah and colleagues and Morest (Nadarajah et al, 2001 and Morest, 1970). When the dextran injection was carried out in the Tg(HuC:GFP) line, monopolar cells in the MZ also expressed HuC:GFP, thus confirming their neuronal phenotype. Single-cell electroporation revealed that the monopolar morphology was a transient phase that preceded the initiation of dendritic connections. Therefore in the zebrafish, newborn neurons lose their ventricle-contacting process and their basal process transforms into an axon, unlike the situations described by the Nadarajah, Morest and Holder papers (Nadarajah et al., 2001, Morest, 1970 and Holder et al., 1991).

Brain slice culture has revealed many aspects of VZ cell behaviour and migration (Tamamaki et al, 2001, Miyata et al., 2001 and Noctor et al., 2004). The recent timelapse study by Noctor and colleagues of rat cortical slices produced several striking observations of VZ progenitor cell behaviour that were hitherto undescribed in such systems (Noctor et al., 2004). The authors showed that many newborn neurons in the VZ undergo a series of four complex migratory behaviours or phases following their birth. During the second phase, newborn neurons adopted a complex multipolar morphology, and the authors describe how "they frequently changed orientation, and extended and retracted processes" within the subventricular zone (SVZ). Even more remarkably, in the final phase of migration multipolar neurons then reverted back to a bipolar morphology and migrated out of the SVZ and back towards the VZ (this

retrograde migration is discussed in further detail in Chapter Five). In their final phase of migration, bipolar neurons were observed to migrate in the orthograde direction, radially into the cortical plate. Additionally, the authors note another remarkable behavioural phenomenon: as the bipolar cells terminated their retrograde migration toward the ventricle and moved in the opposite direction, they appeared to reverse their cellular polarity and transformed their trailing process into a leading process, and what was their leading process during retrograde migration, vice versa. During my dye injection study, no such multipolar cell morphology was ever observed. However, this may be because of the transient nature of this morphology, and the 24-hour gap between observation times. It is curious that although one other study also reports a similar retrograde migration toward the ventricle followed by rapid reverses in cell polarity (Nadarajah et al., 2002) some other authors are unable to detect the transient multipolar morphology or retrograde migration in their own preparations of brain slices (Tamamaki et al., 2001 and Miyata et al., 2001).

One important distinction must be considered when comparing my differentiation data with those of mammalian brain slice culture. The aim of many timelapse studies of cortical slices is to observe the behaviour and differentiation of progenitors during and after cell division, with regards to division symmetry and cell morphology (Tamamaki et al., 2001, Miyata et al., 2001 and Noctor et al., 2004). Such studies have shown that in the cortical slice VZ cell division is the more common neurogenic event (Tamamaki et al., 2001, Noctor et al., 2001 and 2004, and Miyata et al., 2001). In contrast, my study revealed that after 48hpf, neurogenesis in the zebrafish was made up almost entirely of VZ progenitor cells directly differentiating into neurons without dividing. Therefore my hypothesised sequence of events of neuronal differentiation concerns the transition of a single progenitor into a single neuron, whereas the cortical slice studies generally focus on the behaviour of the progeny of VZ cell division.

No Stellate Astrocytes, Oligodendrocytes or Ependymal Cells

It may appear curious that in this study no labelled progenitor cell was found to differentiate into a stellate astrocyte or oligodendrocyte. Voigt showed that in the cortex of ferrets, many radial glia differentiate into stellate astrocytes at perinatal stages (Voigt, 1989) and this had been described in humans, monkeys and the chicken also (Choi and

Lapham, 1978, Schmechel and Rakic, 1979 and Gray and Sanes, 1992) whilst more recently mouse transgenesis has confirmed this (Gaiano et al., 2000, Malatesta et al., 2003 and Schmid et al., 2003). The Gaiano study also showed that some radial glia give rise to ependymal cells. Malatesta and colleagues showed that many oligodendrocytes are derived from radial glia in the mouse forebrain (Malatesta et al., 2003). In my study no stellate astrocytic fate was recorded; however, this result is not surprising since stellate astrocytes have not been described in any teleost brain outside the optic nerve (Nona et al., 1985 and Levine, 1989; for discussion see Kálmán, 1998) other than the sole report of Kawai and colleagues (Kawai et al., 2001). From the antibody staining I performed in the previous chapter, GFAP-positive cell bodies are abundant throughout the A-P length of the spinal cord from 36hpf onwards, although these cells do not possess the complex, multipolar morphology characteristic of stellate astrocytes and are radial in character, reminiscent of those described in the spinal cord of amphibians (Miller and Liuzzi, 1986). In none of my preparations of hindbrain was a GFAP-positive astrocyte with stellate morphology detected (see previous chapter) therefore I believe it is not unreasonable to find that no dextran-injected progenitors in the hindbrain VZ gave rise to such cells.

The lack of oligodendrocytes or their precursors is not surprising either, considering that such cells are generally believed to arise from a medioventral domain in the neural tube and then migrate throughout its entirety. During my studies it was not possible to label these progenitors because of their depth within the brain, therefore I restricted my study to the dorsal ventricular zone only. The characterisation of myelination in the zebrafish by Brösamle and Halpern (Brösamle and Halpern, 2002) demonstrated a comparatively much smaller number of oligodendrocytes in the zebrafish brain in contrast to the mouse (see Calver et al, 1998). Looking at the photomicrographs in the Brösamle and Halpern study, at 48hpf there are approximately ten P0-expressing oligodendrocyte precursor cells, and none expressing myelin-binding protein (MBP), residing in a very ventral domain in the hindbrain. Even by 4dpf, there are probably no more than twenty oligodendrocytes in each rhombomere (see figure in Brösamle and Halpern, 2002). Therefore the mathematical probability of labelling an oligodendrocyte precursor at 48hpf is very small, considering there are five hundred VZ progenitor cells in each rhombomere at 48hpf, and only approximately ten identifiable OPCs in the entirety of

the hindbrain at this stage. Also, in the zebrafish hindbrain oligodendrocytes and their precursors are restricted to a very ventral position, even after the initiation of myelination at 4dpf (Brösamle and Halpern, 2002), unlike the widespread dorsoventral and mediolateral spreading of these cells observed in the spinal cord of mammals. Therefore all evidence weighs against the labelling of oligodendrocytes in this study.

No ependymal cell fate was observed in this study. However, at what stage this cell type first appears is not known, since there are no descriptions of ependymoglia in the zebrafish. Certainly by light microscopy it was not possible to detect any ciliated cuboidal cells, characteristic of ependymoglial morphology, in thick hindbrain sections up to 14dpf. However, ependymal cells probably do develop at some stage beyond this since they have been described in the adult C carpio, a fellow cyprinid (Kálmán, 1998).

In the previous chapter I described a population of GFAP-expressing cells that first appears at approximately 5dpf, and I labelled these cells 'glycogen-rich' or 'mature' radial glia because they were found to lie in the same D-V position as radial glycogen-rich processes and apparent exit from the cell cycle (see previous chapter). In addition to their expression of the differentiated astrocytic marker GFAP these cells possessed an ovoid soma with a long, thin pial-extending process that branches heavily on reaching the marginal zone and before contacting the pial surface. The processes are conspicuous also in terminating in enlarged endfeet. These cells do not incorporate BrdU when treated with a single pulse of BrdU at 5dpf (see Figure 3.7, C, in previous chapter) and have the outward appearance of being a form of differentiated, mature radial glia or radial astrocyte. It was a disappointment then, that no such 'mature' radial glial cells were revealed in this lineage study. There are approximately 500 VZ progenitors in each rhombomere at 48hpf (Lyons et al., 2003) and I labelled 101 clones in my dye injection study. Therefore labelling approximately 20% of the total number of progenitors was probably a fair sample, but not entirely representative of all the cells of the VZ. Also, since these glycogen-rich radial glial cells appear at around 5dpf, their first appearance would not be in the timeframe of my dye injection study, and only using my 1-week tracing technique by electroporation would they be apparent. Since I labelled only a small number of cells (12) by this second method then it is not surprising that I did not visualise these cells. The lineage of these mature radial glial cells therefore remains

unknown. It should eventually be possible to determine their lineage by increasing the number of VZ cells followed the single cell electroporation technique.

Paucity of Cell Divisions

It may be considered unusual that so labelled few cells in my study underwent cell division. Studies of radial glia-like progenitor cells in the mouse have proved these cells to be highly proliferative (Miyata et al., 2001, Noctor et al., 2001, 2002 and 2004, Malatesta et al., 2003 and Anthony et al., 2004). However, these studies are in the mouse forebrain; compared to the number of cells in the zebrafish hindbrain, the population is much greater. The paucity of injected cells that underwent cell division over the 24-hour period of my study is not surprising when one considers a) there are only approximately 2,500 cells in total in each rhombomere at 48hpf, of which at least 80% are postmitotic neurons, and b) the rate of neurogenesis levels off in the hindbrain at around 72hpf (Graph 4.1) (Lyons et al., 2003). My BrdU incorporation analysis in the previous chapter corroborates this also. There is very little cell division after 48hpf simply because by this stage, the hindbrain already possesses most of the cells it requires.

Asymmetric cell divisions were not observed, with neuron-pair symmetric, terminal mitoses comprising all the cell divisions revealed in this study by dextran injection. This further corroborates previously published descriptions of neurogenesis in the zebrafish hindbrain: it has been shown that between neural keel stage and 48hpf, 84% of neurons are born of neuron-pair divisions (Lyons et al., 2003). I also demonstrated in the previous chapter that the number of BrdU-incorporating cells in rhombomeres 4 (r4) and 5 (r5) in the dorsal VZ falls from 40 to 18 between 48 and 72hpf. This represents a decrease of 55%, reflecting the downward shift in proliferation that occurs at this stage in this population. At 48hpf, the start of my experiment, the VZ of r4 and r5 in the hindbrain is composed of approximately 1000 progenitors (Lyons et al., 2003). By my proliferation analysis in the previous chapter, there are on average 40 BrdU-incorporating cells in the dorsal VZ of r4 and r5 at 48hpf (Graph 3.1, Chapter Three). Therefore only 4% of cells in the VZ are in S-phase of the cell cycle at this stage. If it is assumed that 30% of a cycling population will be in S-phase at any one time, 12% of the hindbrain VZ population is still in the cell cycle at 48hpf. The disparity of this

calculated figure with the observed 6% of cells undergoing cell division as revealed in my dye injection study could be explained by my labelling technique. When injecting, I attempted to label randomly in the mediolateral axis in order to sample the whole of the hindbrain dorsal VZ fairly. I have demonstrated in the previous chapter that by 48hpf, in the dorsal VZ BrdU-incorporating cells are largely restricted to the rhombic lip, which lies in a very lateral position. There are comparatively much fewer cycling cells in medial dorsal VZ positions than in rhombic lip. This may explain the lower percentage of observed cell divisions when compared to the frequency of cells in cycle revealed by BrdU incorporation. One way to control for this would be to repeat the labelling experiment, but deliberately target the injections laterally, to the rhombic lip area.

Closing Remarks

In this chapter I have mapped the fate of bipolar, GFAP-expressing radial glia-like progenitors in the hindbrain VZ of the zebrafish from 48hpf onwards and have shown that majority of these cells are directly neurogenic without undergoing cell division. Differentiation into stellate astrocyte-like cells was not observed, nor was self-renewing, asymmetric cell divisions. Cell divisions were rare but those observed were all neuron-pair, terminal divisions. Before this thesis there had been no such studies in any non-mammalian vertebrate system, with the majority of research based on the mouse forebrain. The study provides unique insight into neurogenesis in vivo, in a non-mammalian vertebrate brain.

It is interesting to consider why these radial GFAP-expressing progenitors behave in the ways that I have demonstrated. In my model, there is no stellate astrocyte fate and no asymmetric, self-renewing cell division, instead the cells being neurogenic through either direct differentiation, without division, into neurons, or through a symmetric, neuron-pair terminal division. Generally, following the reports in mouse, neurogenic radial glia are thought to be rapidly proliferative, divide in an asymmetric stem cell-like mode to generate neurons and differentiate into astrocytes perinatum. Certainly the zebrafish hindbrain requires a much smaller total cell number for functionality when compared to the mammalian cortex, which may help explain the low frequency of cell divisions. A surprising result is the lack of asymmetric cell divisions, since almost all current textbooks and reviews regard self-renewing asymmetric cell division as an

important part of vertebrate neurogenesis (Huttner and Brand, 1997 and Wodarz and Huttner, 2003). As I alluded to earlier, repeated rapid, symmetric cell divisions may be better suited to the very short embryonic generation time of zebrafish, whereas the asymmetric method would produce the greater number, and diversity, of neural cells required for the mammalian brain at a slower rate over a longer time period. The lack of stellate astrocytes, a known fate for mammalian radial glia (Voigt, 1989, Gaiano et al., 2000 and Schmid et al., 2003), corroborates previous work in teleost systems (Nona et al., 1985 and Kálmán, 1998) and reflects the lesser variety in cells of the astroglial lineage in many anamniotes (Zamora, 1978, Miller and Liuzzi, 1986 and Bodega et al., 1993).

In Chapter Three I characterised the morphology, marker expression and proliferation of these radial progenitors in fixed tissue. Here I mapped their developmental fate at the level of the single cell. The following chapter makes use of a GFP-expressing transgenic zebrafish line to observe a large group of these cells develop as a population, and visualise the somewhat illusive 'mature' radial glia not observed in this chapter.

Chapter Five

Analysis of Hindbrain Neurogenesis in the Stable Transgenic Tg(zFoxD3:GFP) Line

Introduction

The FoxD3-GFP (FoxD3 was originally described as fkd6; see Odenthal and Nüsslein-Volhard, 1998 and Kelsh et al., 2000) stable transgenic zebrafish line was raised two years ago by Gilmour and colleagues to observe the migration of an unknown peripheral glial subtype during the formation of the lateral line; in this transgenic many cells of the neural crest lineage express GFP during their development (Gilmour et al., 2002). Using this line, Gilmour and colleagues demonstrated how neural crest-derived glia in the developing lateral line migrated concurrently with neuronal axons, and how the axons provided guidance cues to the glia as they migrated to reach their appropriate rostrocaudal position (Gilmour et al., 2002). However, it was subsequently found by our laboratory that a subpopulation of radial progenitor cells in the hindbrain expresses GFP in a stable manner, allowing their observation using *in vivo* microscopy in the intact animal. Genes containing the winged-helix or 'fork head' DNA binding domain first identified in *Drosophila* over a decade ago have been found to be important in the development in many species ranging from yeast to humans (reviewed by Kaufmann and Knöchel, 1996) and nine members of the *fork head* family of transcription factors have been cloned in zebrafish (Odenthal and Nusslein-Volhard, 1998). FoxD3 (fkd6) is a member of the class V group of *fork head* genes, sharing high homology with the mouse BF2 gene. During development FoxD3 is expressed in the floorplate, hindbrain neuroepithelium, neural crest, tailbud, epiphysis and the forming somites (Odenthal and Nusslein-Volhard, 1998). By the standardised transgenic zebrafish nomenclature as encouraged by the Univeristy of Oregon's Z-FIN network (http://zfin.org) the FoxD3-GFP transgenic line as raised by Gilmour and colleagues will be referred to throughout this chapter as Tg(zFoxD3:GFP) and the transgene as FoxD3:GFP.

The utilisation of green fluorescent protein (GFP) isolated from *Aequea vitoria* has provided the cell biologist with a powerful tool for *in vivo* studies. In developmental biology its impact has been considerable as the researcher now is able to watch single or small groups of cells develop and behave naturally – grow, change shape, divide,

differentiate or die – by live imaging alone. Here I use it to observe the fundamental processes of embryonic neurogenesis: progenitor cell division, radial migration of newborn neurons and differentiation. In my previous chapter I described these behaviours at the single cell level by labelling cells with intracellular fluorescent dye; here I will examine the development of a subset of progenitors that are found in the VZ of the hindbrain and express FoxD3:GFP. Expression of GFP in these cells allows the dynamics of a population of cells to be studied as well as giving a much higher resolution of cellular detail than we could see from dextran-injected cells.

Migration of newborn neurons in the embryonic brain is a fundamental process required for the development of a functional CNS. Not only is the number and diversity of neural cells crucial to correct brain function, but their correct position is also, and it has been shown in a variety of species and brain regions that migration, sometimes over large distances, of neuronal cells plays an important role in the patterning of the embryonic brain such as in the cerebral cortex, the retina, the tectum and olfactory bulb (reviewed by Luskin, 1994). The tangential migration of newborn neurons from the ganglionic eminences to cortex to give rise to GABAergic interneurons is one of the most striking examples in the brain (O'Rourke et al., 1992, Tan and Breen, 1993 and Anderson et al., 1997), but radial migration i.e., from ventricular or germinal zone to mantle layer, is the more global event taking place at almost all A-P levels of the CNS. For example, although it has been studied most in the mammalian cerebral cortex, radial migration has also been described in the cerebellum (Rakic, 1971b and Edmondson and Hatten, 1987), chicken telencephalon and optic tectum (Gray and Sanes, 1992 and Szele and Cepko, 1998) and the cerebellum of the teleost *Apteronotus*, the ghost knifefish (Clint and Zupanc, 2002). A most striking defect in radial migration is the *reeler* mouse, defective in the *reelin* signalling pathway, where newborn neurons in the cortex are incapable of radial migration in an 'inside-out' mode and arrange themselves in a 'rim-to-core' pattern instead (see Caviness and Rakic, 1978). However, despite being such a widespread and common phenomenon in the developing brain, to date there is no description in the literature of radial migration in a living, intact embryo: the radial migration at the cortex of *Macacus* described by Rakic (Rakic, 1971a and 1972), the ghost knifefish (Clint and Zupanc, 2002) and in the chick tectum and forebrain (Gray

and Sanes, 1992 and Szele and Cepko, 1996 and 1998) were all described in fixed tissue. The Tg(zFoxD3:GFP) zebrafish transgenic line combines photostable, GFP-expressing neural progenitors with rapid development and optical transparency, and offers an excellent opportunity to observe radial migration in a living, healthy vertebrate embryonic brain.

The aims of this chapter follow on from the results of the previous two. In Chapter Three I characterised ventricular zone (VZ) progenitors using antibody markers, and in the preceding chapter I performed a lineage analysis of these cells using fluorescent tracers. Here I have the necessary tools to observe VZ progenitors, as a population in a timelapse manner. Firstly, I will observe how cells migrate out of the VZ and into the mantle zone (MZ). This process, whilst described in many systems in the literature, has never been observed *in vivo*, in the intact brain. Second, by observing FoxD3:GFP-expressing cells as a quantifiable population, the progressive depletion of the embryonic VZ with development, and differentiation of its progenitors, will be examined. Lastly, the orientation of cell division in the hindbrain VZ will be recorded by timelapse microscopy.

Methods & Materials

Confocal microscopy

Laser scanning confocal microscopy (LSCM) was necessary to resolve unambiguously the complex population of GFP-expressing cells within the developing hindbrain. Both live, anaesthetised transgenic Tg(zFoxD3:GFP) embryos and fixed animals manually sectioned with a microsurgical knife were imaged. Some specimens were immunostained with antibodies to GFAP, GFP, acetylated tubulin or HuC/HuD protein (see Table 3.1, Chapter Three) and then imaged. A fixed-stage Leica confocal microscope under the control of Leica TCS NT software (Version 1.6.587, Leica Microsystems, Heidelberg) was used to capture z-series of images that were saved as tagged-image file format (TIFF) files and exported to a MacIntosh G4 computer for analysis. The images and series were processed using NIH Image, Image J and Adobe Photoshop (Version 7, Adobe Systems Inc, San Jose).

Timelapse microscopy

I developed a protocol for live imaging of GFP-expressing cells in the posterior hindbrain in the Tg(zFoxD3:GFP) transgenic line, photographing the embryos for up to 14 hours. Since the zebrafish develops from a zygote to larva in approximately three days, this period covers a substantial proportion of late embryonic brain development. Thirty-two to 36hpf transgenic embryos were anaesthetised with MS-222 Tricaine, set in 1.5% low melting point agarose and mounted so that they would be imaged from the lateral aspect. Taking up the embryo in a drop of molten agarose, it was placed on a thickness 1 22mm x 22mm glass coverslip and rapidly oriented with a blunted tungsten needle. Then the coverslip was inverted and set onto a chamber of embryo medium made by drawing a small circle of water-repellent silicone grease onto a microscope slide. Therefore the embryo would be held in place in a sealed, safe environment for the duration of the timelapse experiment. The embryo medium inside the chamber also contained MS-222 Tricaine to keep the embryo anaesthetised and 0.003% PTU to retard melanocyte differentiation. The timelapse microscopy was carried out using a Zeiss Axioplan 2 microscope with Hamamatsu Orca ER CCD camera and both microscope and camera were controlled by the software Openlab (Version 3.1.5, Improvision UK, Coventry). Openlab possesses an 'Automator' function which captures images for timelapse automatically at specified time intervals for the duration of the experiment. For all the timelapse experiments, images were captured every three minutes in a single focal plane using a 63x water immersion objective lens. The ambient temperature of the microscope room was maintained at approximately 28°C by using a portable oil heater. It was found to be necessary to leave the embryo in its agarose chamber for approximately one hour to stabilise in the mountant prior to image capture; following this settling in period the embryos would develop quite normally and healthily. Since during this period the brainstem of the zebrafish becomes thickened and the body undergoes a 'straightening' as the head-trunk angle decreases the GFP-expressing cells of interest would often move very slightly in different planes so I found it necessary to readjust the focus or area of interest as the timelapse proceeded. After approximately twelve to sixteen hours of timelapse microscopy from a start point of 32hpf, the restriction of growth by the agarose tends to retard the embryo and makes videos of

longer than sixteen hours impractical, however, the naturally rapid speed of growth in the zebrafish meant this period was equivalent to approximately 20% of the total period of embryonic development, and one quarter of embryonic neurogenesis, starting at the time when the rate of hindbrain neurogenesis is at its peak (see Lyons et al, 2003).

Multiphoton Timelapse Microscopy

The timelapse videos made with the epifluorescence of the Zeiss Axioplan were very informative but since the images were only captured in one optical plane, interference from fluorescent cells above and below the level of focus rendered many parts of the timelapse indistinguishable. Likewise, the cells of interest were very mobile and migrating radially, making them move out of the plane of focus as development proceeded. To counter this, I decided to make use of the new multiphoton microscopes available to us here in the department. The embryos were prepared for the timelapse as described in the previous paragraph, but instead were imaged using a Leica Multiphoton Microscope.

Quantification of GFP-Expressing Cells

The number of GFP-expressing cells in the hindbrain was quantified with respect to developmental age. At regular intervals from 24hpf to 14 days post-fertilisation (dpf) live Tg(zFoxD3:GFP) embryos were live imaged from the dorsal by aspect by LSCM, and then the GFP-expressing cells counted using the Disector Method (Sterio, 1984) as described in Chapter Two, whilst displayed in the program NIH Image.

Immunofluorescence Antibody Staining

Embryos for immunohistochemistry were dechorionated, heavily anaesthetised with tricaine and fixed in 6% paraformaldehyde (PFA, Sigma) in PBS (phosphate-buffered saline, Sigma) overnight at 4°C. The primary antibodies used in this project are listed in Table 3.1.

Wholemount antibody staining was carried out according to fairly standard zebrafish protocols (see Westerfeld, 1995):

15. Remove from fixative and wash with PBS.

16. Dehydrate with a methanol-PBS series into 100% methanol, store at –20°C overnight.
17. Return to room temperature and rehydrate with a PBS-methanol series until the embryos are in 100% PBS.
18. For embryos older than 36hpf, embryos permeabilise with 0.25% trypsin (Sigma) on ice. (Embryos were generally treated for between 10 – 30 minutes).
19. Wash 3 x 5 minutes in PBS and refix with 5% PFA for 20 minutes at room temperature.
20. Wash off fixative with a 5-minute wash in PBS.
21. Place embryos in 3 x 5 minutes washes in PBTr (PBS with 1% Triton X-100 (Sigma))
22. Serum block with 10% normal goat serum (Sigma) in PBTr for one hour at room temperature on shaker.
23. Dilute antibodies in 10% serum and add to embryos. Incubate embryos at 4°C on a shaker overnight.
24. Return to room temperature and wash in PBTr on a shaker for four hours, changing the solution every 20 minutes. (Antibodies frequently used were recycled once after use).
25. Dilute secondary antibodies (species-specific fluorophore-conjugated antibodies (Alexa-488 (green) or Alexa-568 (red), Molecular Probes)) 1:200 in 10% serum and add to embryos. Incubate overnight at 4°C on shaker.
26. Return to room temperature and wash in PBTr on a shaker for four hours, changing the solution every 20 minutes.
27. Post-fix in 5% PFA for 20 minutes at room temperature.
28. Mount in 50% glycerol-PBS for imaging.

Results

A Subpopulation of Radial Cells in the Hindbrain VZ Express GFP in the Tg(zFoxD3:GFP) Transgenic from 24hpf Onwards

FoxD3:GFP-expressing cells are first detectable in the hindbrain at approximately 24hpf. When observed from the lateral aspect, these cells are large, bipolar and contact both basal and apical extents of the developing neuroepithelium, at this stage the hallmark of neuroepithelial cells (Figure 5.1, B and C). GFP-expressing cells are restricted in the A-P axis to rhombomeres 5 and 6, and to the anterior spinal cord. As neurogenesis continues the neural tube thickens and expands, and these cells maintain their bipolar morphology whilst their cell bodies become progressively restricted to the dorsal ventricular zone (VZ)(Figure 5.1, D - F). Their basal processes extend radially to the pial surface (Figure 5.1, D - F). From 30hpf onwards another population of GFP-expressing cells is observed to lie in the mantle zone (MZ) often associated with or in close proximity to the basal processes of VZ cells (Figure 5.1, D - F).

Stereotyped Mediolateral Patterning of FoxD3:GFP-Expressing Cells

When viewed in transverse section it is apparent that the FoxD3:GFP-expressing cells are restricted to a distinct mediolateral position within the VZ (Figure 5.2). From approximately 32hpf, GFP-expressing cell bodies are clustered just medial to the lateral edge of the VZ (Figure 5.2, B – E). This position, just medial to the rhombic lip, is maintained throughout development, and the basal processes of these cells in the VZ radiate outwards to the pial surface in relatively tight bundles, reminiscent of the GFAP-expressing fibres of the glial curtains (Figure 5.2, and see Chapter Three). In the mantle zone (MZ), GFP-expressing cells are associated with basal processes, and some possess neuronal morphology such as rounded or polygonal soma (Figure 5.2, E – F). Between 4dpf and 5dpf, the basal processes of VZ cells become elaborately branched, on reaching the marginal zone (Figure 5.2, F and G). The branched processes also possess enlarged or rounded endfeet in contact with the pia. This complex structure is reminiscent of 'mature' or 'classic' radial glia as described in the literature previously, and identified at this stage by GFAP antibody staining in Chapter Three.

Figure 5.1. FoxD3:GFP-expressing cells in the caudal hindbrain during embryonic and larval development.

A. *Camera lucida* diagram of 36hpf embryo, adapted from Kimmel et al, 1990, with inset showing the area of interest magnified in B – E. B – E are Tg(zFoxD3:GFP) transgenic embryos live imaged from the lateral aspect by LSCM, all these images are maximum intensity projections of confocal z-series.

B. 24hpf. GFP expression reveals large, bipolar cells with endfeet contacting both ventricular and pial extremes of the hindbrain, hallmarks of neuroepithelial cells.

C. Green fluorescence image of B superimposed over transmitted light, showing the ventricular (VS) and pial (PS) surfaces of the neural tube (NT). Occasional GFP expression outside the brain is from neural crest derivatives such as undifferentiated melanocytes and peripheral neurons (asterisks).

D. 36hpf. With neurogenesis proceeding rapidly at this stage, the DV thickness of the hindbrain becomes greatly increased and the size of neural cells decreases; however, the cells populating the dorsal ventricular zone maintain their bipolar phenotype, their pial processes becoming lengthened and remaining in contact with the PS. GFP-expressing cells in more ventral positions are in close proximity with pial processes.

E. 48hpf. The dorsal ventricular zone is greatly diminished by this stage, comprising only three to four cell thicknesses at the dorsalmost extreme of the hindbrain, whilst many GFP-expressing cells are scattered in the mantle layer.

F. 72hpf. The GFP-expressing population becomes compacted and restricted in the A-P axis as cells in the anterior spinal cord and rhombomere 5 no longer express the transgene. The cells in the dorsal ventricular zone continue to express GFP strongly, whilst the GFP expression in cells in the mantle layer appears to decline; the D-V dimension of the neural tube continues to expand as the axon tracts of the marginal zone develop. By this stage GFP expression is no longer detectable in the periphery.

Scale bar: 100μm.

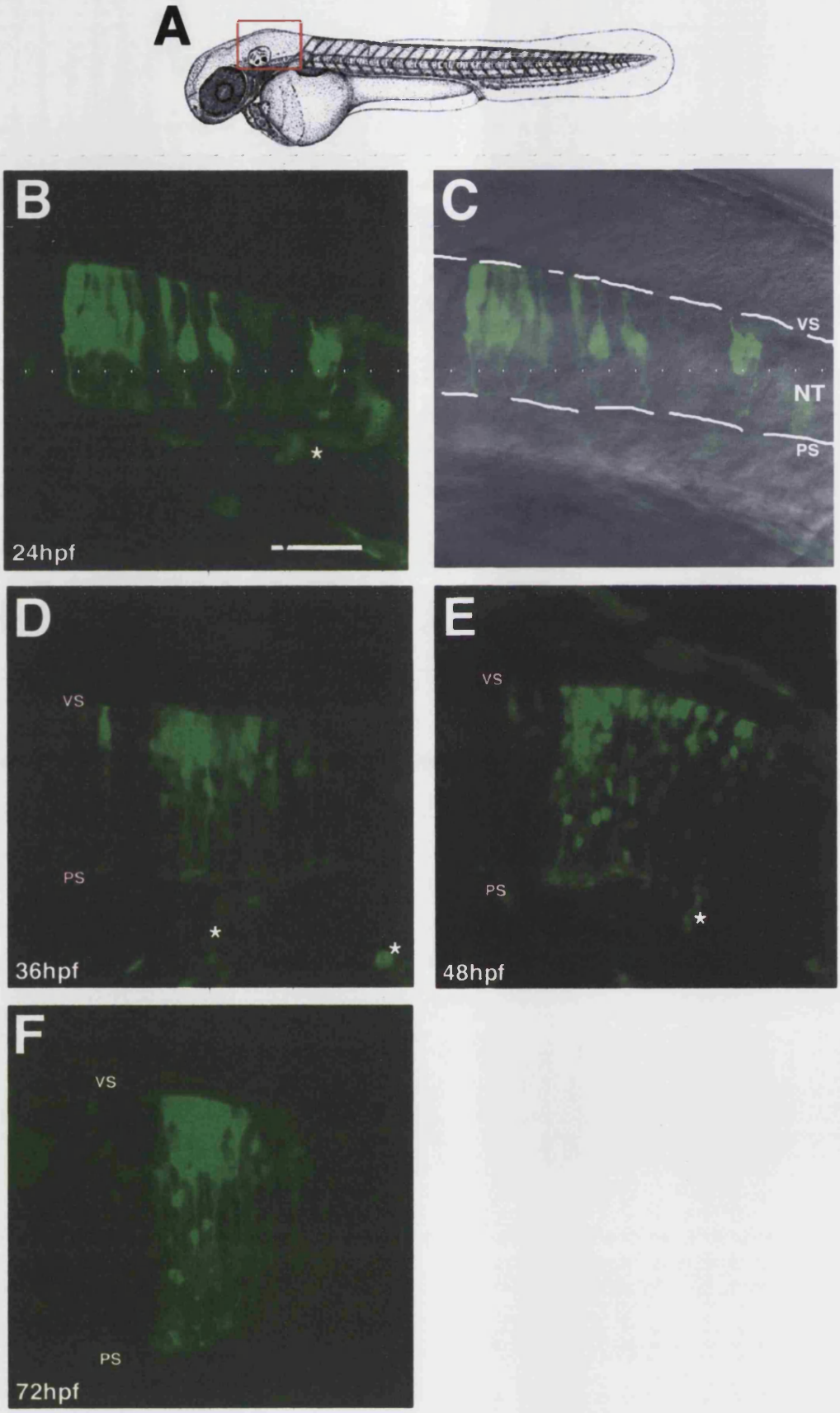
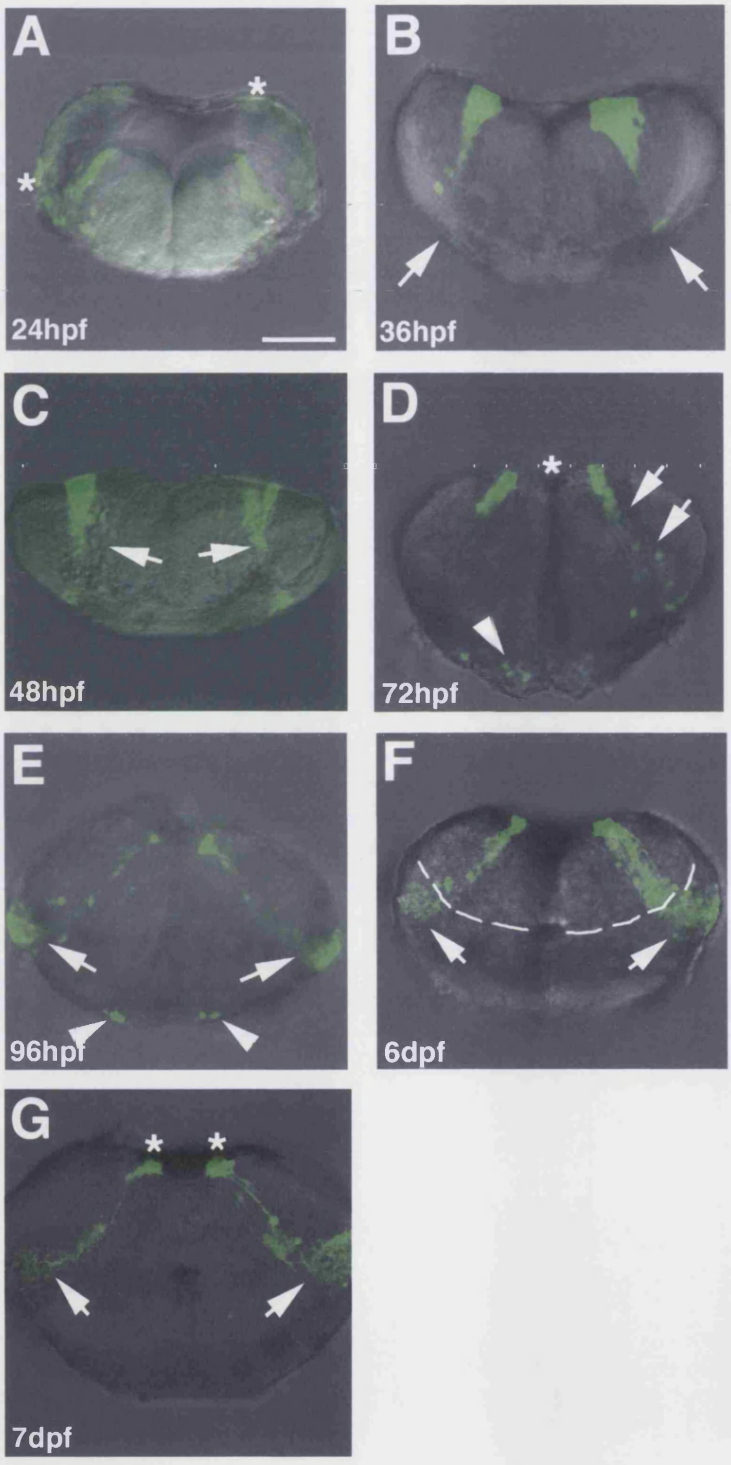

A
B
24hpf
C
VS
NT
PS
D
VS
PS
36hpf
E
VS
PS
48hpf
F
VS
PS
72hpf

Figure 5.2. FoxD3:GFP-expressing cells in the caudal hindbrain during embryonic and larval development. Transverse section.
Maximum intensity projections of caudal hindbrain transverse sections imaged by LSCM; the images are fluorescence projected onto transmitted light.
A. 24hpf. GFP-expressing progenitors within the brain are large and span the D-V length of the brain, with typical neuroepithelial cell morphology. There are GFP-expressing cells in the periphery due to neural crest derivatives such as undifferentiated melanocytes and peripheral neurons and glia also expressing the transgene (asterisks).
B. 36hpf. With development the cross-sectional area of the brain increases, and the cell bodies of GFP-expressing progenitors become largely restricted to the dorsal ventricular zone (VZ), although they project their basal processes to the pial surface (arrows). Occasional cell bodies with neuronal morphology begin to be detectable in the ventral grey matter.
C. 48hpf. GFP-expressing cell bodies become more frequent in the mantle layer (arrows), but they express GFP more faintly than the cells in the VZ.
D. 72hpf. The GFP-expressing cell bodies in the dorsal VZ are much reduced in number by this stage, and faint profiles of monopolar cell bodies are still distinct in more ventral positions (arrows). These cells appear to be aligned in radial units corresponding to the pial processes of the dorsal VZ cells, implying they are migrating from dorsal VZ into the mantle layer along these radial processes. Sporadic GFP-expressing cells are notable in the ventral marginal zone (arrowheads). The distance between the cells in the two halves of the brain decreases as the VZ appears to converge on the midline (asterisk; compare with C).
E. 96hpf. The cells at the dorsalmost region of the hindbrain are greatly reduced in number by this stage, with greater GFP expression in pial processes, especially the endfeet (arrows), and cell bodies in the mantle zone. The VZ becomes increasingly restricted medially. Bilateral groups of GFP-expressing cell bodies continue to be visible in the marginal zone (arrowheads).
F. 6dpf. The radially extending pial processes belonging to the small number of GFP-expressing cells still at the dorsal ventricular surface become elaborate and branched (arrows), particularly at the interface between mantle layer and marginal zone (broken line). These processes permeate through the axon tracts of the marginal zone and terminate at the glia limitans. Many GFP-expressing cell bodies are still visible in the grey matter of the mantle zone, nestled in and amongst these complex radial fibres.
G. 7dpf. By this stage the GFP-expressing cell bodies in the mantle layer have almost entirely disappeared, leaving behind the small number of persistent radial cells with their cell bodies lying at the dorsal ventricular surface (asterisks). Elaborate branched radial processes in the axon tracts of the marginal zone are apparent (arrows).
Scale bar: 100μm.


A
24hpf
B
36hpf
C
48hpf
D
72hpf
E
96hpf
F
6dpf
G
7dpf

Population Dynamics of the Transgene-Expressing Cells

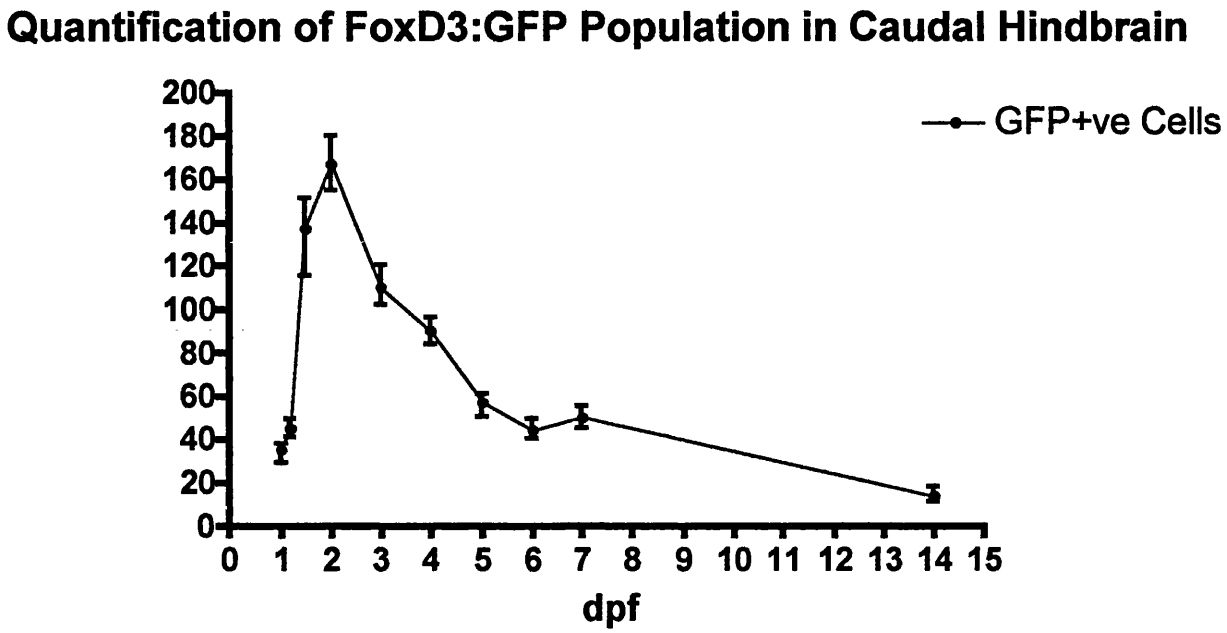
The total number of FoxD3:GFP-expressing cells in the hindbrain was counted to quantify the dynamics of this population (Graph 5.1). Following their first appearance in the hindbrain at around 24hpf, the population increases dramatically, almost tenfold, over a twenty-four hour period, peaking at 48hpf with a total of nearly 170 cells (Graph 5.1). From this stage the population declines steadily up to the seventh day of development. Between 36hpf and 4dpf, the transgene-expressing cells are found in large numbers in both the VZ and MZ. However, by 7dpf most GFP-expressing cells are restricted to the VZ and the MZ population is no longer detectable (Figure 5.1 and 5.2). From 7dpf to 14dpf, the VZ population also declines greatly, from an average of fifty cells down to only fifteen. Throughout development, no left-right asymmetry in cell numbers was observed.

Cell Death Partly Accounts for the Decline in FoxD3:GFP Population

From 72hpf through to 7dpf dying cells were observed at the midline of the VZ throughout the A-P length of the hindbrain, including the caudal region where the FoxD3:GFP population resides (Figure 5.3, A – D). These cells were extruded from the neural tube and protruded into the fourth ventricle, and possessed a characteristic rounded, vacuolated appearance (Figure 5.3, A – D). Some of these dead cells appeared to be the remnants of GFP-expressing cell bodies (Figure 5.3, D). This pattern of death may account at least partially for the marked reduction in the number of FoxD3:GFP-expressing population following the second day of development. This localised cell death may be apoptotic, although this is yet to be confirmed.

FoxD3:GFP-Expressing Cells in the Mantle Zone Are Neurons

Although many GFP-expressing cells reside within the VZ, between 36 and 48hpf many are found to lie within the mantle zone (MZ) (Figure 5.1 and 5.2). Several lines of evidence demonstrate that these cells are differentiated neurons (Figure 5.4). Firstly, immunohistochemistry shows that these cells express HuC/HuD protein (Figure 5.4, A and B). Second, antibody staining to GFP in fixed transverse sections shows that these cells possess both cell body morphology and axonal projections characteristic of neurons



Graph 5.1. Quantification of GFP-expressing cells in the caudal hindbrain of Tg(zFoxD3:GFP) embryos

Embryos were live-imaged by LSCM and the number of GFP-expressing cells in the hindbrain quantified. Each timepoint is the total number of cells expressing GFP in caudal hindbrain region. No lateral asymmetry was observed; each count is the bilateral total number of GFP-expressing cells. All specimens were counted by the Disector Method (Sterio, 1984) as described in Chapter Two. Each timepoint is the mean of three specimens, with the vertical bars representing the range.

Figure 5.3. Cell Death of FoxD3:GFP-expressing cells during late embryonic and early larval development

Tg(zFoxD3:GFP) embryos live-imaged from the dorsal aspect by LSCM. Images are maximum intensity projections of z-series.

A. 72hpf. A small number of rounded-up, vacuolated cells extruded from the neuroepithelium become visible by transmitted light microscopy (arrowhead). These cells are invariably at the dorsal midline.

B. 7dpf. The number of dying cell bodies at the dorsal midline has increased greatly by this stage. Bi: Transmitted light showing bunched dead cells at the ventricular surface (arrowhead). The darkened patches are an artefact of imaging caused by pigment-containing melanocytes in the skin of the animal. Bii: green fluorescence showing the FoxD3:GFP-expressing radial glial cells.

C. Higher magnification view of GFP-expressing cells in B, Ci: green fluorescence, Cii: green transmitted light. Many of the dead cells are GFP-expressing radial glia.

D. Detail of area indicated by asterisk in C. Top: green fluorescence shows rounded-up, dying GFP-expressing cells. Middle: transmitted light illustrates the characteristic morphology of these dying cells. Bottom: overlay of the two channels.

Scale bar: 100μm.

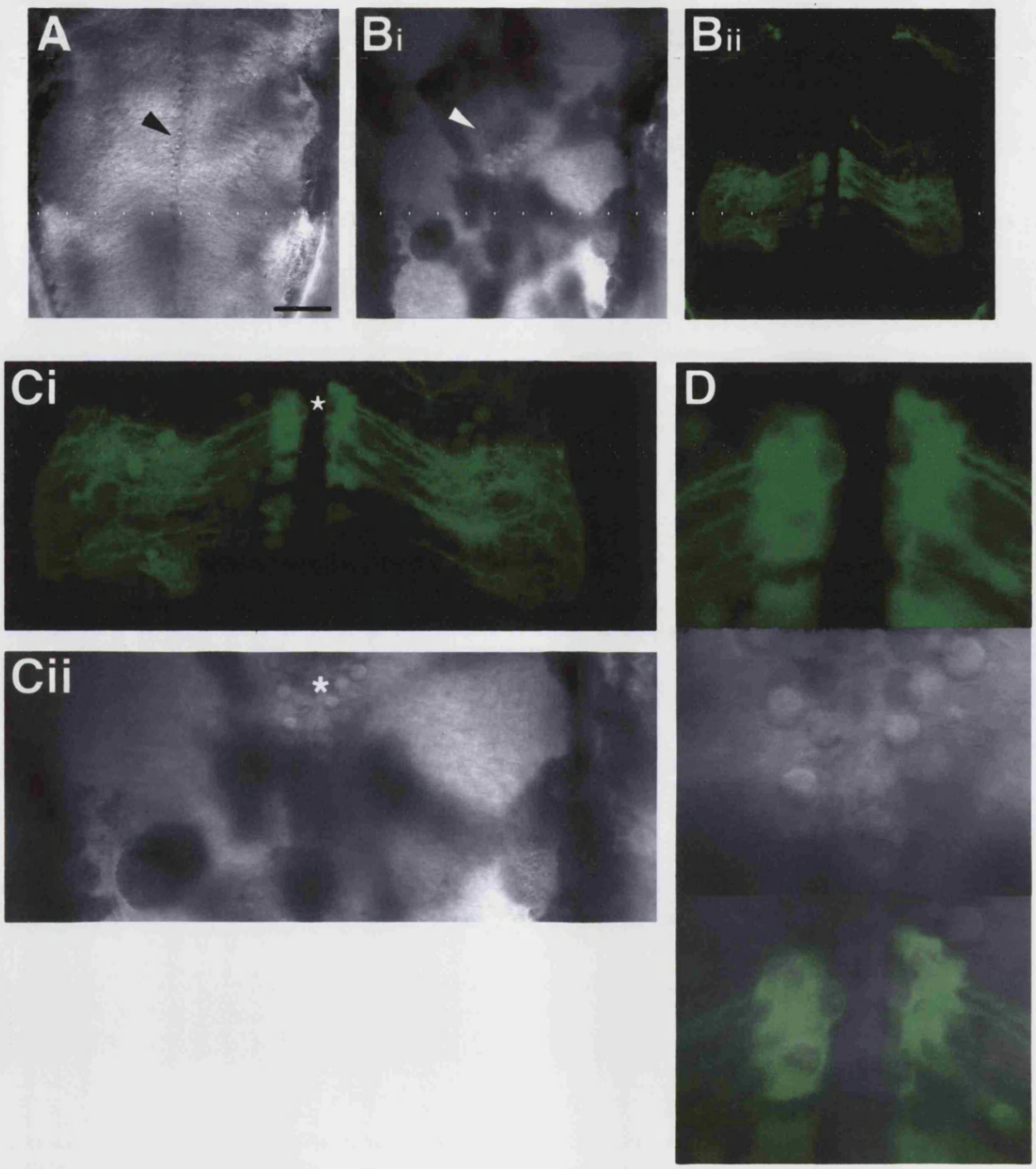
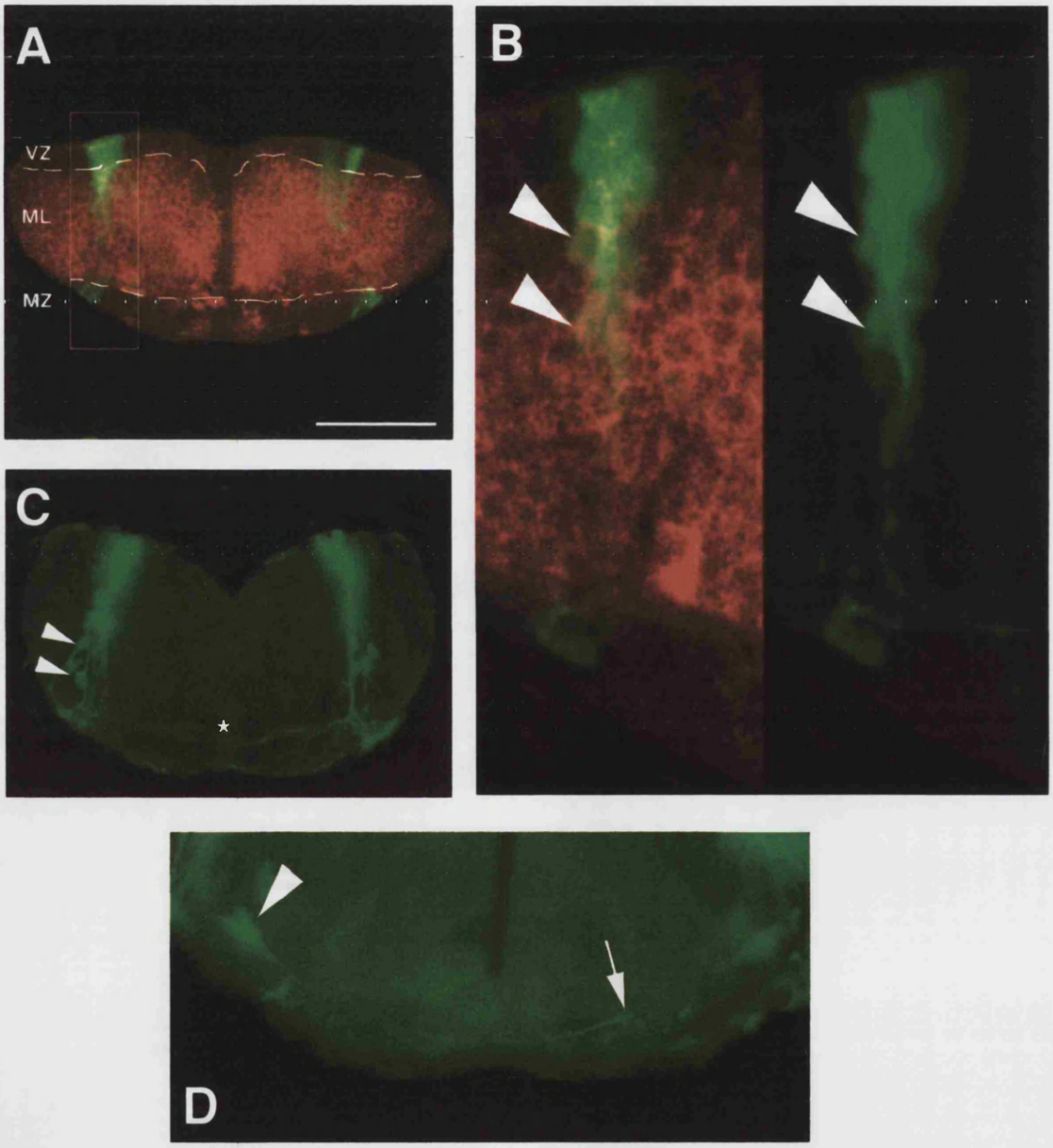

A
Bi
Bii
Ci
Cii
D

Figure 5.4. FoxD3:GFP-expressing cells differentiate into neurons.
Marker expression (A and B) and morphology (C and D) show that many GFP-expressing cells differentiate into neurons.
A and B: 48hpf Tg(zFoxD3:GFP) transgenic embryos stained with antibodies to GFP (green) and HuC/HuD protein (red), a marker of postmitotic neurons.
A. Transverse section of the caudal hindbrain imaged by LSCM and rendered as a maximum intensity projection. The three laminae of the hindbrain – ventricular zone (VZ) composed of progenitors, mantle layer (ML) of neuronal cell bodies and the white matter of the marginal zone (MZ) – are delineated by HuC/HuD protein expression. Note the processes belonging to dorsal GFP-expressing cell bodies extend to the pial surface and terminate in endfeet.
B. Single confocal section taken from the inset in A, showing that GFP-expressing cells in the process of migrating radially from the VZ into the ML express the HuC/HuD protein (arrowheads).
C. 36hpf. Maximum intensity projection of a confocal z-series imaged by LSCM in transverse section. Several GFP-expressing cells in the ventral grey matter (arrowheads) have clear axonal projections such as commissural axons (asterisk).
D. Single confocal section of a separate specimen showing a GFP-expressing cell body (arrowhead) extending its axon across the midline. This morphology is characteristic of commissural interneurons.
Scale bar: 150μm.

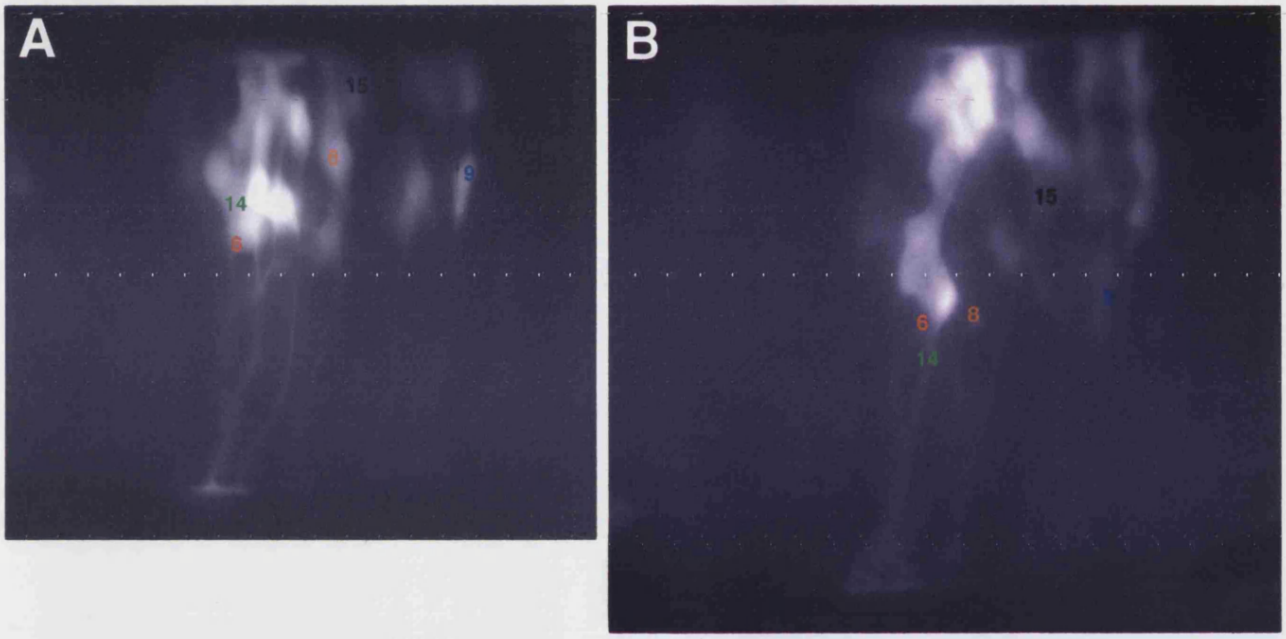

A
VZ
ML
MZ
B
C
D

(Figure 5.4, C - E). Many of these neurons have commissural axons, but their precise identity and subtype is unknown.

Timelapse Video Microscopy Reveals Radial Migration of FoxD3:GFP-Expressing Cells

Timelapse video microscopy of the caudal hindbrain between 32 and 54hpf, from the lateral aspect, reveals the complex and dynamic nature of neurogenesis in the context of an intact brain. The following observations are made from Supplemental Movies 1 – 3 (see attached CD-ROM). At 32hpf GFP-expressing cells are generally concentrated in the dorsal VZ, and as development progresses some of these cells migrate in a radial manner to a more ventral position, in the MZ. These radially migrating cells use the basal processes of VZ cells as a substrate for their ventral migration into the MZ. Individually, migrating cells are seen to contact numerous basal processes as they migrate. A large proportion of these cells' surface area is in intimate contact with such processes, and they appear to exert considerable mechanical tension onto the radial fibres which are seen to bend and buckle and be temporarily deformed in shape by migrating cells and fall back into shape afterwards. Migration is saltatory, with migrating cells often dithering in their movement, or even migrating slightly in a dorsal direction, partway through their ventral motion (Figure 5.5). Examination of the basal processes below the VZ reveals a high level of filopodial activity. Fine tangential bridges of cytoplasm are seen to extend and collapse rapidly between processes (Figure 5.6). The basal processes in the marginal zone, terminating at the pial surface, also appear to be quite dynamic during the course of development. By analysis of the timelapse movies it was possible to quantify the speed of radial migration as displayed by the GFP-expressing cells during neurogenesis. The net velocity in the ventral direction, i.e., including the periods of migratory arrest, exhibited by GFP-expressing cells was calculated as 2.7 – 4.4$\mu m.hour^{-1}$ with a mean of 3.48$\mu m.hour^{-1}$ (n=5). The velocity of actual cell migration, i.e., not including the periods of migratory arrest, was 9.6 - 20$\mu m.hour^{-1}$, mean 13.2$\mu m.hour^{-1}$. This figure is much lower than previously published reports of radial migration in mammalian *in vitro* cortical slice systems, which describe mean velocities of 19.7 ($\pm$6.2) $\mu m.hour^{-1}$ (Noctor et al, 2004) and 27 ($\pm$2.4) $\mu m.hour^{-1}$ (Gongidi et al, 2004). Modelling the radial migration of cerebellar granule

Figure 5.5. Radial migration is saltatory in Tg(zFoxD3:GFP) hindbrain
From Supplemental Movie Two. Embryo is imaged from the lateral aspect, with anterior to the left, as per described in the Materials and Methods section.
Five cells that underwent orthograde radial migration were tracked using the 'Measurements' tool in the Improvision program Openlab, between 34hpf (A) and 44hpf (B). The starting and final location of the cells is labelled. Cell body position was labelled every 15 minutes and plotted as a cell track (C). By the appearance of the tracks alone, it would appear there is also a slight caudal (left to right) migration of both these cells, however, this is drift due to the straightening of the zebrafish body axis during this period of development, and is not a real migration (see Supplemental Movie Two). The radial migration of GFP-expressing cells is saltatory: it is composed of short and rapid migrations in the ventral direction punctuated by periods of migratory arrest. This appears in the cell track as bunches or groups of timepoints separated by short straight tracks. Over a period of 9.4 hours, the cells migrated on average 35.2μm in the ventral direction, at a net velocity of 2.7 – 4.4μm.hour^{-1} with a mean of 3.48μm.hour^{-1}. Discounting the periods of migratory arrest, the velocity of the cell migration alone was 9.6 – 20.1μm.hour^{-1}, with a mean of 13.2μm.hour^{-1}. Individual velocities (net and of migration only) are as follows: Cell 6: 2.7 (10.0), Cell 8: 4.3 (15.0), Cell 9: 4.4 (9.6), Cell 14: 2.8 (11.4) and Cell 15: 3.2 (20.1) μm.hour^{-1}.


A
15
8
9
14
6
B
15
6
8
14

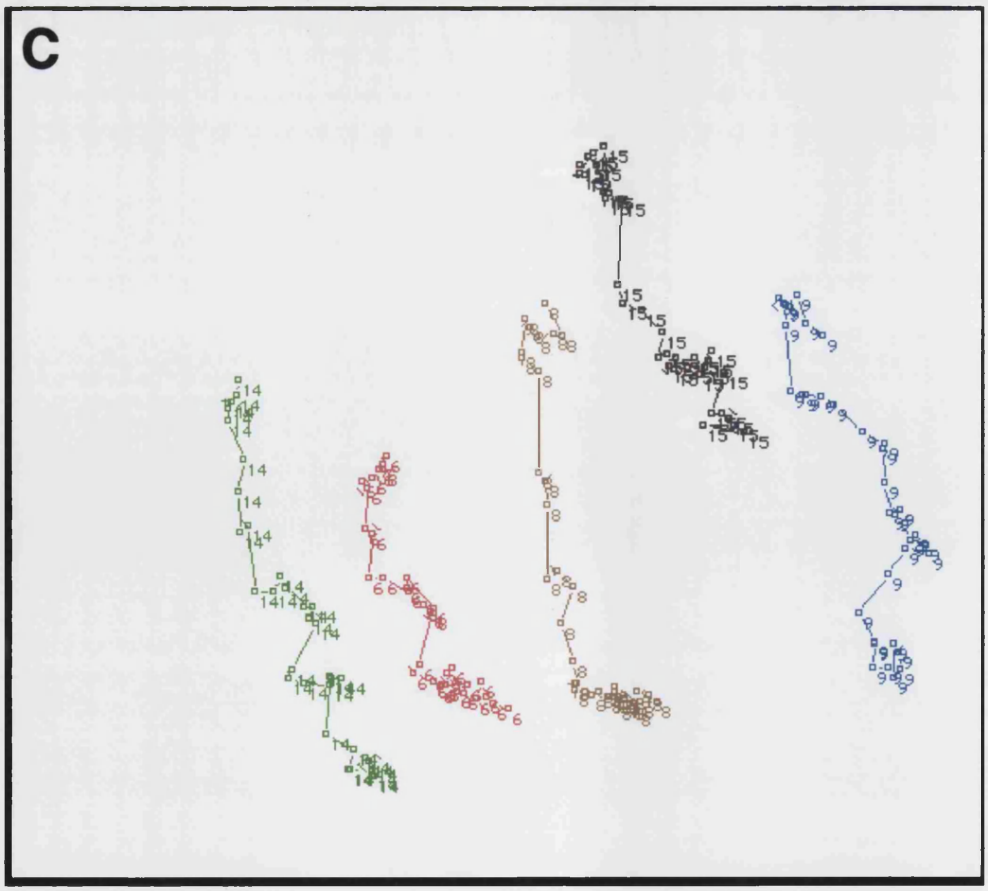

C

Figure 5.6. FoxD3:GFP-expressing cells and basal processes exhibit a dynamic morphology and structure.

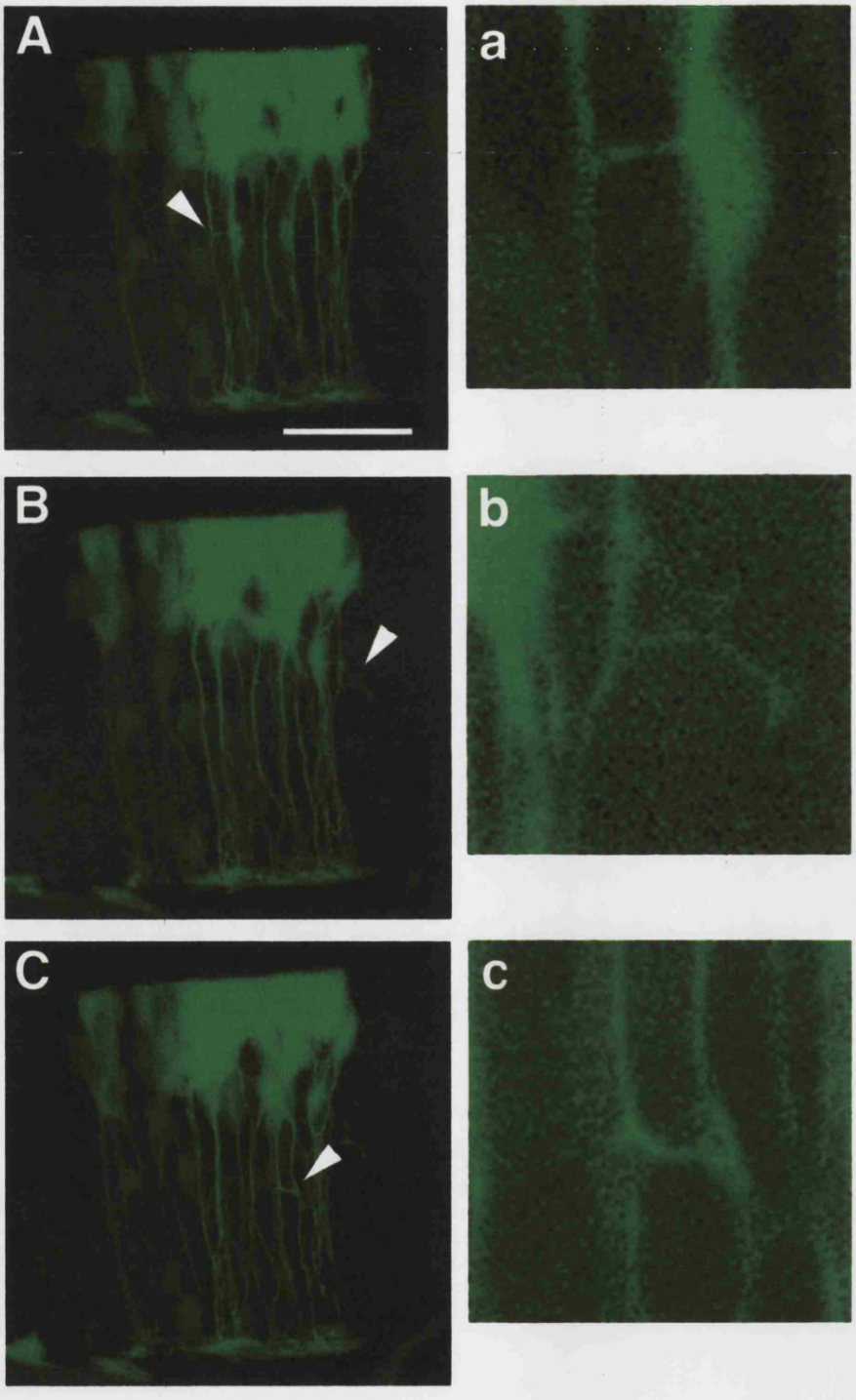
Timelapse imaging by multiphoton microscopy reveals extension and collapse of filopodia between cells and basal processes. Maximum intensity projections of multiphoton z-series, live-imaged from the lateral aspect with anterior to the left. The timelapse was started at 38hpf. Arrowheads in A - C denote the magnified region of interest in a – c.

A. t=15'. Lateral filopodial extension from a migrating neuron to a separate basal process.

B. t=90'. Branched filopodium extending into the posterior GFP-negative region.

C. t=130'. Thick filopodial bridge formed between two basal processes.

Scale bar: 100μm.


A
a
B
b
C
c

neurons on Bergmann fibres *in vitro* microcultures, Edmondson and Hatten report a velocity of 33 (±20) μm.hour^{-1} (Edmondson and Hatten, 1987).

FoxD3:GFP-Expressing Cells Use GFAP-Expressing Processes As a Substrate For Radial Migration

Tg(zFoxD3:GFP) embryos were fixed and stained with an antibody to GFAP. This showed that the radially oriented basal processes on which MZ cells migrate express GFAP (Figure 5.7). When viewed from the dorsal aspect, it becomes clear that most, it not all, GFP-expressing cell bodies in the MZ are in contact with at least three GFAP-expressing processes (Figure 5.7, A and B). Using the program 'Volocity' to 3D-render antibody-stained transverse sections imaged by LSCM, it is clear that FoxD3:GFP-expressing cell bodies in the MZ are migrating through a 'forest' of radially oriented, GFAP-expressing processes (Figure 5.7, C).

Retrograde Migration of GFP-Expressing Cells

Analysis of the timelapse movies revealed a minority of GFP-expressing cells that, having migrated out of the VZ and into a more ventral position in the mantle layer, then migrated very rapidly in the reverse direction, back into the dorsal VZ. This migration was marked in its rapidity, calculated to be 20.0 – 39.8μm.hour^{-1}, with a mean of 32.67μm.hour^{-1}, which is more than twice the velocity of orthograde migration (Figure 5.8). See Supplemental Movie Two to see this phenomenon. It should be noted that this migration is not interkinetic nuclear migration, since BrdU incorporation studies in Chapter Three have shown that S-phase cells are never observed at levels this deep in the MZ. Therefore radial migration toward the ventricle will be referred to as retrograde migration, and that toward the pial surface will from here be referred to as orthograde migration. It was unfortunate that retrograde-migrating cells could not be followed further after migrating back into the VZ, since they tended to become lost in the group of GFP-expressing cells in the VZ. This behaviour may be similar to that reported recently in rat *in vitro* cortical slice culture (Noctor et al., 2004). Here Noctor and colleagues showed that the radial migration of neurons born in the VZ often contained a phase of retrograde migration from the subventricular zone (SVZ) back into the VZ as a prelude to final radial migration into the cortical plate.

Figure 5.7. FoxD3:GFP-expressing cells migrate radially along GFAP-expressing processes.

48hpf Tg(zFoxD3:GFP) transgenic embryos stained with antibodies to GFP (green) and GFAP (red).

A. Single confocal section of the caudal hindbrain imaged from the dorsal aspect by LSCM. GFP-expressing neuronal progenitor cell bodies are surrounded in their circumference by GFAP-immunopositive fibres. Aii. Closeup of inset in A, showing how the majority of radially migrating GFP-expressing cells are in close contact with at least three GFAP-expressing fibres.

B. Single confocal section of a transverse section imaged by LSCM. The basal processes of FoxD3:GFP-expressing cells extend to the pial surface. The broken line indicates the D-V level of the confocal section in A, just below the ventricular zone.

C. Three-dimensional rendering of the z-series imaged in B, using Improvision software 'Volocity'. Radially migrating GFP-expressing cell bodies are surrounded or encased in three dimensions by multitudinous fine, GFAP-positive fibres (arrowheads). The lateral GFAP-sparse regions correspond to clustered cell bodies (CB) of the hindbrain commissural interneurons; their axons (CA) projecting across the midline are strongly GFAP-positive, most likely due to neurons inheriting GFAP from their immediate predecessors. Some GFP-positive endfeet are visible at the pial surface (arrows).

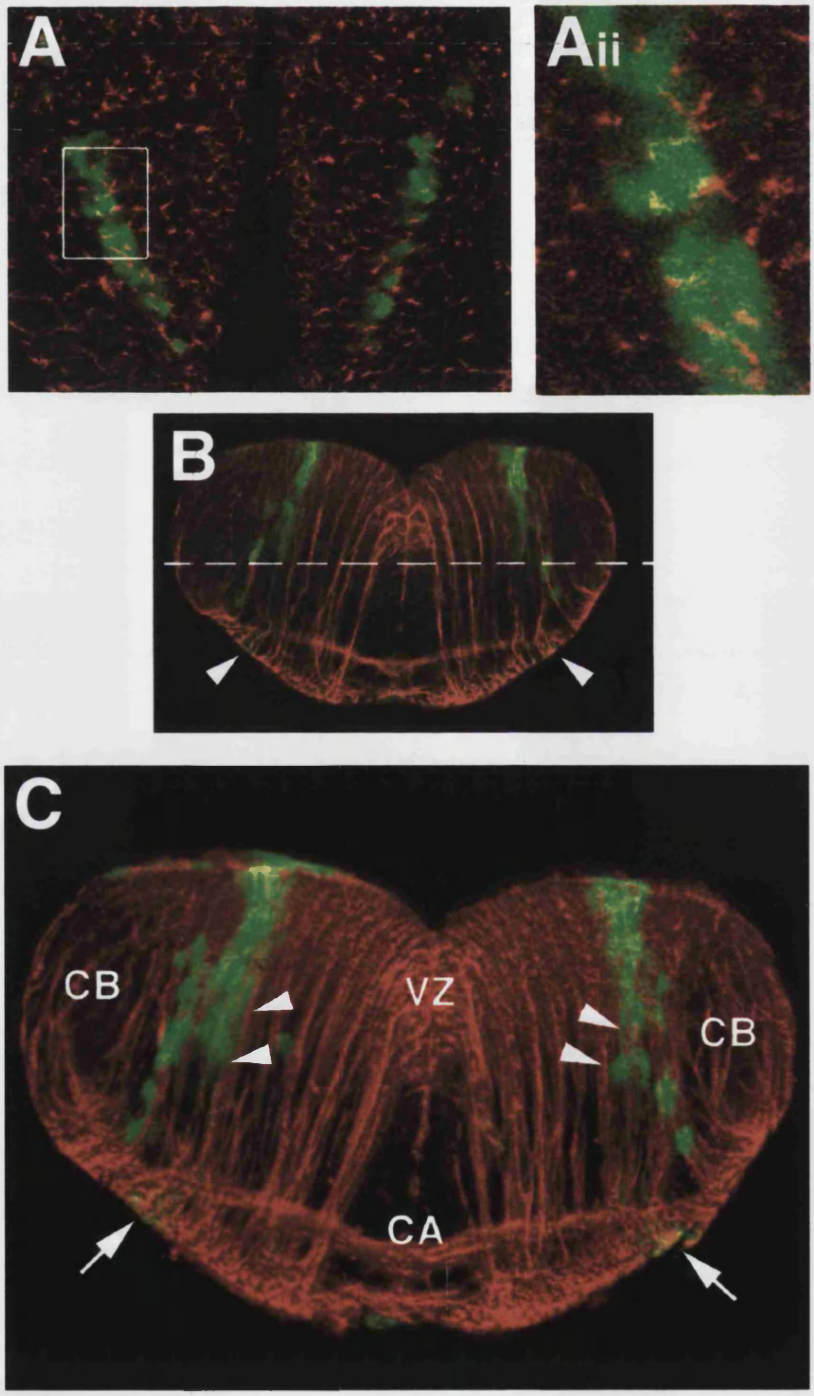
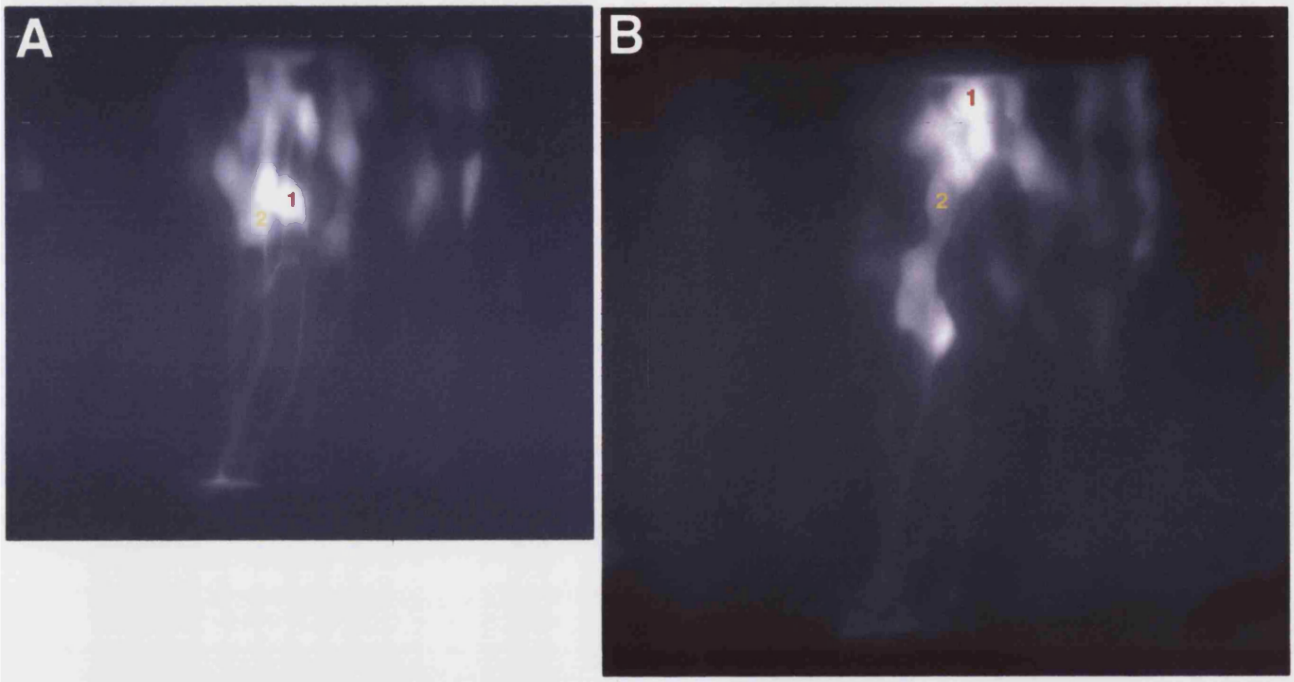

A
Aii
B
C
CB
VZ
CB
CA

Figure 5.8. Retrograde radial migration

From Supplemental Movie Two. Embryo is imaged from the lateral aspect, with anterior to the left, as per described in the Materials and Methods section.

Two cells that displayed retrograde migration were tracked using the 'Measurements' tool in the Improvision program Openlab, between 34hpf (A) and 44hpf (B). The starting and final location of both cells is labelled. Cell body position was labelled every 15 minutes and plotted as a cell track (C). Both cells show an initial orthograde radial migration, and then turn back toward the ventricle. The orthograde migration of Cell 2 shows a classical saltatory mode of migration, but Cell 1 displays a rather steadier movement. By the appearance of the tracks alone, it would seem there is also a slight caudal migration of both these cells, however, this is drift due to the straightening of the zebrafish body axis during this period of development, and is not a real migration (see Supplemental Movie Two). The velocity of retrograde migration as calculated as 20.0 – 39.8$\mu m.hour^{-1}$, with a mean of 32.67$\mu m.hour^{-1}$ (n=3). Not only is this velocity two to three times that of orthograde migration, but this movement is not saltatory, as revealed by following the cell track. The retrograde migration of Cell 1 returns it to the ventricular surface, whilst Cell 2 appears to stop short within the ventricular zone.


A
1
2
B
1
2


C

Low Frequency of Cell Division in GFP-Expressing Cells After 32hpf

Timelapse microscopy of ventricular zone FoxD3:GFP-expressing radial progenitors in the posterior hindbrain shows that after 32hpf the majority of these VZ cells differentiate without division into unipolar cells with the appearance of neurons. In five movies, totalling altogether over 75 hours of recorded timelapse, only four cell divisions were observed to take place within the FoxD3:GFP population. In every case the cells divided within the plane of the ventricular surface of the brain, a mode of cell division that is generally described as 'horizontal' or 'parallel' to the plane of the ventricular surface (Figure 5.9). No cell division perpendicular to the ventricular surface was ever observed by timelapse microscopy.

These data corroborate the findings of the previous chapter that show that VZ progenitor cell division after 48hpf was a comparatively rare event. Furthermore, a single pulse of BrdU at 48hpf shows that less than 2.6% of GFP-expressing cells are in S-phase of the cell cycle (Figure 5.10).

Discussion

Tg(zFoxD3:GFP) transgenic as a tool to observe neural development in vivo

In this chapter I have used a stable transgenic zebrafish line to visualise neurogenesis during late embryonic development and radial gliogenesis in the early larva. Although the Tg(zFoxD3:GFP) line was raised to observe neural crest derivatives a small subpopulation of hindbrain progenitors express GFP and I was able to observe and make timelapse movies of neurogenesis as it occurred in a healthy, unperturbed embryo. In addition to the corroboration of much of the data of the previous chapter, the visualisation of radial migration *in vivo* is the first of its kind to date and highlights the utility of the zebrafish as a model organism in investigations of developmental neurobiology.

Figure 5.9. Horizontal cell divisions revealed in timelapse movies of developing Tg(zFoxD3:GFP) embryos

An example of FoxD3:GFP-expressing cell undergoing cell division at approximately 36hpf.

A. Original still from timelapse movie, with inset indicating the area magnified; ventricular surface (VZ) and pial surface (PS) are labelled. These stills are taken from Supplemental MovieTwo.

B. Close-up of inset in A. Colours have been artificially inverted.

t=0: the cells is rounded up and lies at the ventricular surface in preparation for cell division;

t+3' the cell cytoplasm becomes pinched around its circumference as a prelude to cleavage;

t+6' cell division is almost complete;

t+9' cell division is complete three minutes later. The two daughters remain alongside one another, both parallel to the plane of the VS, throughout the course of cell division.

C. As B, but with the cell profiles outlined.

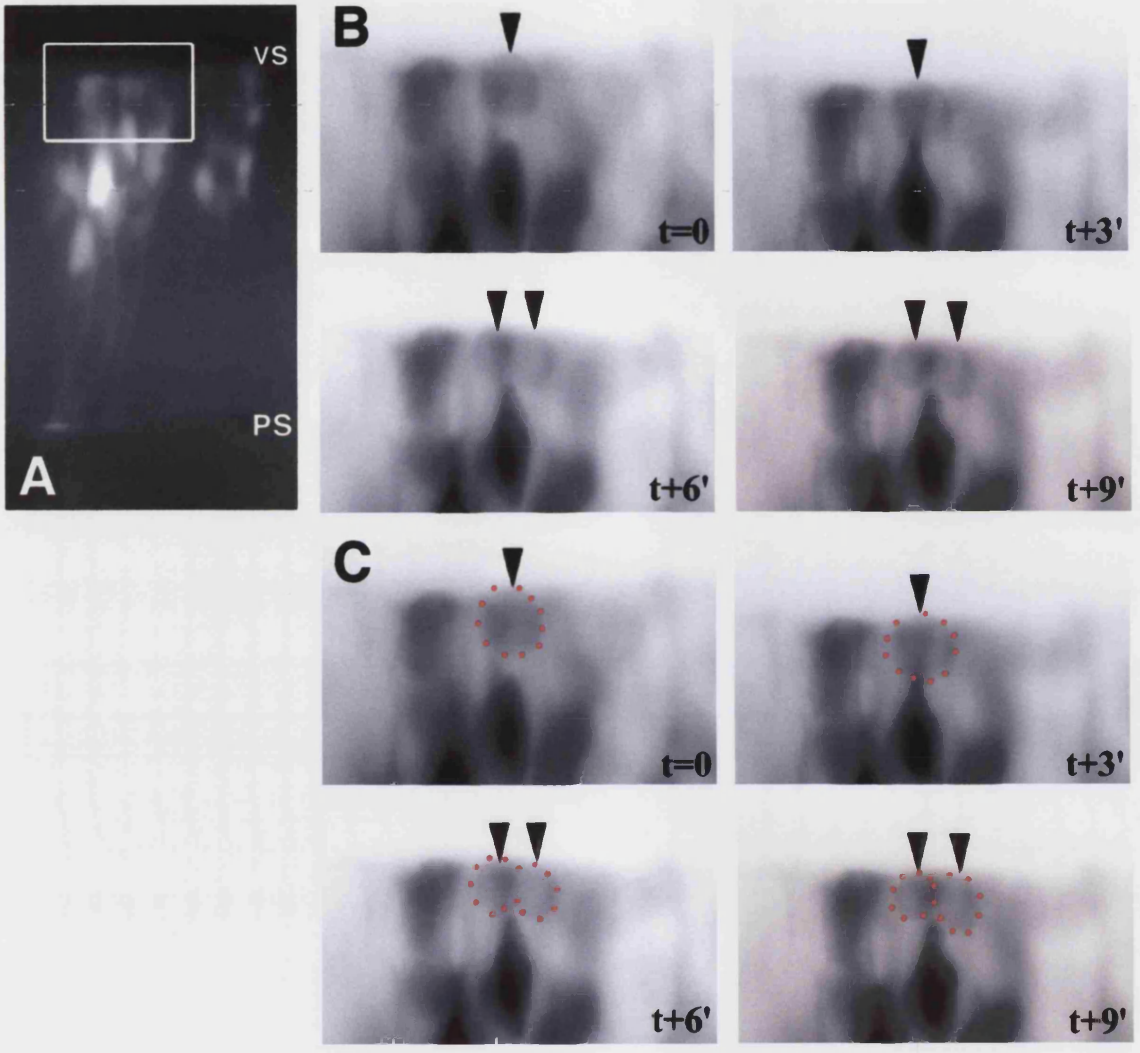

A
VS
PS
B
t=0
t+3'
t+6'
t+9'
C
t=0
t+3'
t+6'
t+9'

Figure 5.10. The majority of FoxD3:GFP-expressing cells are not in S-phase of the cell cycle by 48hpf

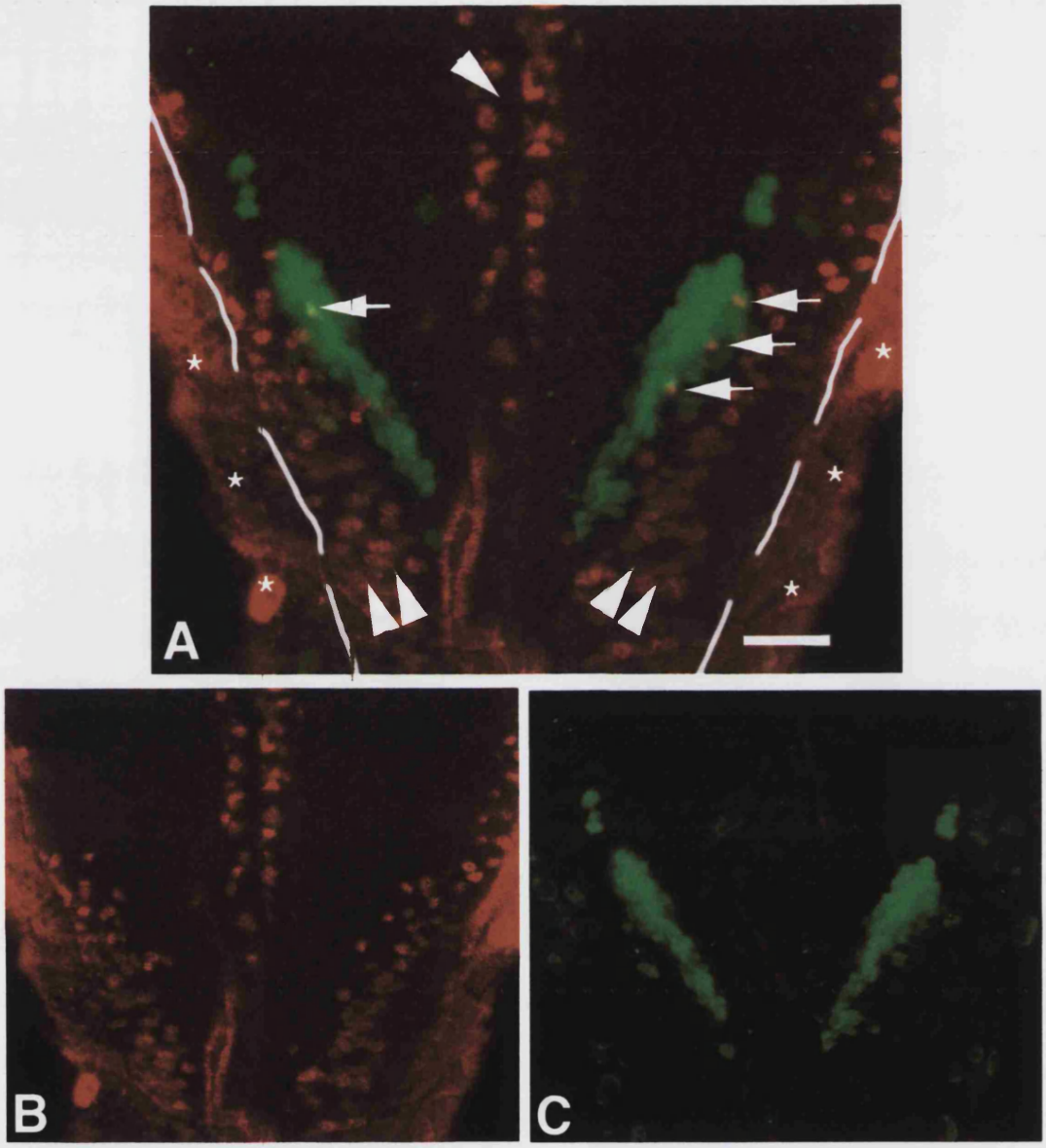
48hpf Tg(zFoxD3:GFP) transgenic embryos were pulsed with BrdU and allowed to recover for 30 minutes before sacrifice and detection of BrdU incorporation by immunohistochemistry. Specimens were imaged by LSCM. The broken line delineates the lateralmost extreme of the neural tube at this stage, as there is some background staining in the skin and periphery (asterisks).

A. Maximum intensity projection of a confocal z-series, imaged from the dorsal aspect (anterior is top). As shown in Chapter Three, by this stage the majority of BrdU-incorporating cells are restricted to a medioventral domain (arrowhead) and the dorsal rhombic lip (double arrowheads). Only a tiny minority, 4/155 (2.58%), of FoxD3:GFP-expressing cells have incorporated the DNA analogue (arrows).

B. Red fluorescence only.

C. Green fluorescence only.

Scale bar: 100μm.


A
B
C

Guiding Radial Migration

After Rakic's descriptions of radial glia in the embryonic cortex of *Macacus* the textbook concept is that radial glia provide a 'guiding rail' for migrating newborn neurons (Rakic 1971a, b and 1972 and Levitt and Rakic, 1980; for review see Rakic, 1990). Recent timelapse studies of mouse cortical slice cultures also appear to corroborate this (Tamamaki et al, 2001, Noctor et al, 2002 and 2004 and Miyata et al, 2002). Here I have shown that a similar system may be present in the hindbrain of zebrafish, but since it was not possible to visualise the morphology of single migrating cells, the mechanics of their migration and the degree of guidance or dependence upon GFAP-expressing fibres are not completed determined. However, this study is certainly the first visualisation of radial migration in the intact, living vertebrate brain. Fixed tissue cannot reveal the dynamic manner in which living cells are behaving, and the live-imaging I performed here produced two novel observations of radial migration. Firstly, instead of the strict stoichiometry of one radial process:one cell system as first described by Rakic (Rakic 1971b and Rakic, 1972) each migrating FoxD3:GFP-expressing cell appears to be in contact with a number of processes in its circumference; antibody staining revealed that most cells were in proximity with at least three GFAP-positive radial processes. Secondly, both the radial processes and migrating cells showed a high degree of plasticity in their morphology, rapidly extending and withdrawing filopodia and processes to each other. To date the only published descriptions of radial migration have been in fixed and stained tissue (e.g., Rakic 1971b and 1972, Levitt and Rakic, 1980, Gray and Sanes, 1991 and Clint and Zupanc, 2001), brain slice culture (Nadarajah et al, 2001 and 2002) or *in vitro* dissociated cells (Edmondson and Hatten, 1987, Gasser and Hatten 1990a and b). In this chapter I reported the average velocity of radially migrating cells as 3.48μm.hour^{-1}. This is the first description and measurement to date of radial migration in the *in vivo* situation. Noctor and colleagues calculated rat newborn neurons to migrate at an average velocity of 19.7μm.hour^{-1} in cortical slice culture (Noctor et al, 2004) whilst Gongidi and colleagues (Gongidi et al, 2003) give a figure of 27 (±2.4) μm.hour^{-1} in a similar system in the mouse. Modelling the radial migration of cerebellar granule neurons on Bergmann fibres *in vitro* microcultures, Edmondson and Hatten report a velocity of 33 (±20) μm.hour^{-1} (Edmondson and Hatten, 1987). It is likely that the much higher velocities reported by Edmondson and Hatten are a result of

the cells migrating in a dissociated culture environment, since in brain slices and *in vivo* the migratory neurons must negotiate a much more dense and complex territory.

Somal Translocation and Glia-Guided Locomotion

Treating mouse cortical slice cultures with vital dyes and recording timelapse movies, Nadarajah and colleagues described two modes of radial migration in the mammalian forebrain, 'somal translocation' and 'glia-guided locomotion' (Nadarajah et al, 2001). According to the authors, somal translocation is apparently a mode of migration that is independent of radial glia (Nadarajah et al, 2001 and Nadarajah and Parnavelas, 2002). This is because serial electron microscopy has revealed that neither the cell bodies nor the short leading processes of translocating cells are in contact with radial glial processes (Shoukimas and Hinds, 1978), two morphologies that are characteristic of glia-guided migration. Also, the migration of labelled somal translocating cells was steady, not saltatory (Nadarajah et al, 2001). The authors also go on to report that earlier-born neurons migrate by somal translocation, whilst later-born neurons migrate via glia-guided locomotion (Nadarajah, 2001, Nadarajah and Parnavelas, 2002 and Nadarajah, 2003). This switch in mode of migration is apparently in response to the increasing complexity and apicobasal expansion of the cortex during neurogenesis. It was not possible to tell from my timelapse videos alone the mode of migration being used by the GFP-expressing cells. Due to the expression of GFP in so many cells in a group, it was not possible to make out the apical and basal processes of single cells. However, combining the observations from timelapse movies here with data from single dextran-labelled cells in the previous chapter, the mode of migration of zebrafish newborn neurons appears to have characteristics of both kinds of migration. According to the literature, saltatory cell movement is characteristic of glia-guided migration (Edmondson and Hatten, 1987, Gasser and Hatten, 1990a and b and Nadarajah et al, 2001), as is the contact of migrating cells with radial, GFAP-expressing processes (Levitt and Rakic, 1980). But looking at the morphology revealed by single-cell dextran injection as shown in Chapter Four, many of the cells seem to be maintaining contact with the marginal zone as they migrate radially, and some possess an apical process that extends toward the ventricle, both morphologies associated with somal translocation, a mode of migration considered independent of glia (Nadarajah et al, 2001, Nadarajah and

Parnavelas, 2002 and Nadarajah, 2003). Nadarajah speculates that glia-guided locomotion is a recent evolutionary adaptation to the greater complexity and thickness of the mammalian neocortex, whilst somal translocation is "an older and simpler mode of movement" (Nadarajah and Parnavelas, 2002 and Nadarajah, 2003). My results suggest that the initial event for many differentiating neurons in the zebrafish may be somal translocation that takes the cell body out of the VZ, and that beyond this point the differentiating neurons could use glia-guided migration through the mantle layer to reach their appropriate final position. However, the conclusions that can be made from my data are limited by the fact that I did not visualise single migrating cells within the group of FoxD3:GFP-expressing cells. In the future, use of Kaede protein, a form of GFP which can be photoconverted by UV light into an RFP, should make it possible to visualise small numbers of migrating cells to fully realise their morphology (Ando et al., 2002 and P. Bayley, personal communication). Also, the laboratory of Tom Schilling is currently raising a transgenic zebrafish expressing GFP under the control of the GFAP promoter, so using dye injection or electroporation in this line it would be possible to simultaneously visualise migrating cells and radial glial fibres, a feat that the Nadarajah studies did not perform.

It is not certain whether in the zebrafish, newborn neurons are dependent on GFAP-expressing radial processes to migrate through the mantle zone, as suggestive of glia-guided migration, but this may be testable in the future. Unpublished observations from the Clarke lab show that laser ablation of a distal portion of a radial FoxD3:GFP-expressing process leads to degeneration of the whole process, so it may be possible to selectively eliminate radial processes locally (personal communication, J. Clarke) and then observe whether new neurons are still able to migrate through the mantle zone.

On Retrograde Migration

The retrograde migration displayed by a minority of cells in the timelapse videos was a most interesting observation. When I first observed this there was no published description of such a phenomenon. Indeed, many impartial observers of my movies believed it an artefact of imaging, due to the repeated and extensive exposure to UV light throughout the course of the timelapse leading to photo-damage of the fluorescent cells. At the time I was fairly confident of this being real, due to two considerations:

firstly, GFP is probably the most stable and least phototoxic intracellular label there is, and secondly, from my experience with single cell dye injection, cells that become apoptotic or die after labelling are extruded from the neuroepithelium into the fourth ventricle very rapidly and are clearly visible in the ventricular fluid. In my timelapse videos none of the retrograde-migrating cells were observed be extruded or become rounded and apoptotic, therefore I believe they are unlikely to be photo-damaged cells. It was only recently that the work of Noctor and colleagues reported a similar retrograde or reverse migration in their cortical slice culture system (Noctor et al., 2004), thereby further indicating that this in fact a real phenomenon. Noctor and colleagues' timelapse study of GFP-labelled radial glia in cortical slices provided some of the first timelapse observations of mammalian VZ progenitor cell behaviour over multiple rounds of cell division. The authors described how neurons born in the VZ undergo four distinct phases of migration. First, they migrate soon after they are born into the subventricular zone (SVZ). The second phase is a period of 'migratory arrest' where the neurons remain in the SVZ, followed by a third phase of retrograde migration back towards the ventricle into the VZ and concluded by the terminal phase of radial migration into the cortical plate. It is this third phase of reverse or retrograde migration that is interesting, since it is in some ways similar to the reverse migration displayed by some cells in my timelapse movies. However, the authors report that the majority of single cells labelled in the VZ displayed this behaviour (approximately 65%) whereas in my movies, it was only a very small minority of FoxD3:GFP-expressing cells that migrated in this fashion. The behaviour of these FoxD3:GFP-expressing cells following their retrograde migration e.g., if they undergo orthograde radial migration back into the mantle zone as in the rat cortex, is not known. It is unlikely that retrograde migrating FoxD3:GFP-expressing cells are returning to the ventricular surface to undergo cell division for two reasons: First, divisions were not observed following the retrograde migrations and second, data in this chapter such as the BrdU analysis and timelapse movies show that cell division is a very rare event after 32hpf.

Another form of retrograde radial migration has been reported in the literature recently. Labelling single neurons with vital dyes in mouse cortical slice culture, Nadarajah and colleagues describe 'ventricle-directed migration' (Nadarajah et al., 2002). Here, timelapse imaging revealed GABAergic interneurons migrating toward the ventricle in

the neocortex. It was well established previously that cortical interneurons are born in the ganglionic eminence and migrate tangentially into the cortex (O'Rourke et al., 1992, Tan and Breen, 1993 and Anderson et al., 1997) but this was the first study to show how, on reaching the cortex from the ventral telencephalon, such interneurons migrate out of the intermediate zone and cortical plate and toward the VZ, with some extending a process to contact the ventricle itself. The velocity of this migration was rapid ($50\mu m.hour^{-1}$) and the authors showed that this retrograde migration was followed by radial migration back into the cortical plate. They also state that the radial migration back towards the pia was slower than ventricle-directed migration, although they did not quantify this velocity. The authors speculated that migrating interneurons use this ventricle-directed migration to gain positional information, to ensure that they come to rest in the correct layer and position before fully adopting their final interneuron morphology. In general, in the fish hindbrain there is very little tangential or rostrocaudal migration of neurons, and in the FoxD3:GFP cells in particular this has never been observed. It would thus seem unlikely that the retrograde migration of FoxD3:GFP cells is required to attain positional information cues.

Notch Signalling and Radial Glia

Another area for consideration is Notch intercellular signalling between radial processes and migrating cells. During development a balance must be maintained whereby even though some cells of the VZ are differentiating into neurons, other cells are prevented from doing so in order to maintain the progenitor population. It has been shown that in the mouse forebrain radial glial processes express Notch1 protein (Gaiano et al., 2000) and migrating newborn neurons in close association with these processes express Notch ligands (Campos et al., 2001). In the cerebellum, Notch1 is expressed in the radial fibres of Bergmann glia at P6, when granule cell migration is at its peak (Patten et al., 2003). Several authors have hypothesised that migrating Delta-expressing neurons may be inducing or maintaining radial glial phenotype via activation of Notch signalling in the cell on whose process they are migrating (Patten et al., 2003, Campos et al., 2001 and Gaiano et al., 2000; reviewed by Gaiano and Fishell, 2002). In the chick retina, progenitor cells express Notch whilst newborn, migrating neurons express Delta1 (Dll) and the resultant intercellular signalling maintains the balance between progenitors and

differentiation throughout development (Henrique et al, 1997). Overexpression of Dll in the progenitors arrested neurogenesis as all cells receive lateral inhibition, whilst removal of lateral inhibition by dominant negative-Dll causes all retinal progenitors to differentiate into neurons (Henrique et al, 1997). It would be intriguing to examine if the same process was taking place in the FoxD3:GFP-expressing radial VZ cells and the newborn neurons migrating radially along their processes. Such experiments are ideally suited to the model system I have used here, i.e., to be able to electroporate constructs into the GFP-expressing cells in the Tg(zFoxD3:GFP) transgenic line and live-image the effects to see if increased Notch signalling would change the balance between progenitors and differentiated cells. In particular, it would be interesting to see it if it were possible to perturb the balance between neuronal and differentiated radial glial fate in the FoxD3:GFP population. In the normal situation only 9% of transgene-expressing cells at 48hpf go on to differentiate into mature radial glia, with the remainder differentiating into neurons, or dying. It has previously been shown in the zebrafish retina that activation of Notch promotes the generation of cells with the characteristics of Müller glia (Scheer et al, 2001). Therefore it would be interesting to see whether activated Notch can direct FoxD3:GFP-expressing cells towards the differentiated, mature radial glial fate (see review by Gaiano and Fishell, 2002).

The Ontogenesis of Radial Glia in the Zebrafish Hindbrain

Radial glia remain an enigmatic cell type in the vertebrate CNS. There are volumes of papers on the subject in mammals and other vertebrates yet in the zebrafish however, there is very little literature regarding this glial cell type. Although there have been descriptions of larval and adult radial glia in other fish species, none has addressed the embryonic situation and certainly none attempted to follow these cells, as a population, through embryonic and larval development. Using the Tg(zFoxD3:GFP) transgenic line I performed a unique characterisation of a population of hindbrain progenitors, from their origins as neuroepithelial cells in the early neural tube to their ultimate fates of either neurons or mature radial glia. The great majority of the original FoxD3:GFP-expressing cells are neurogenic during embryogenesis, whilst less than 9% are fated to differentiate into a form of mature astrocytic radial glial cell in larval stages of life. The persistent and stable expression of the GFP transgene in these cells allowed me to live-

image these cells, characterise their marker expression and quantify the population over time.

As should be apparent from the data in this chapter, FoxD3:GFP-expressing cells in the embryonic hindbrain share many of the characteristics of radial glia as described in the literature over the years. Their bipolar morphology with ovoid cell body lying in the ventricular zone (VZ), the expression of the intermediate filament marker GFAP and the migration of newborn, nascent neurons along their basal processes all corroborate previous work (for reviews see Rakic, 2003 and Weissman et al, 2003). However, there are a number of interesting characteristics in the behaviour of these cells that set them apart from previously described radial glia. Firstly, FoxD3:GFP-expressing cells are apparent in the neuroepithelium as early as 24hpf, and these cells have the bipolar morphology of neuroepithelial cells. These cells are, by morphology, outwardly indistinguishable from all other neural progenitors at this stage. As development progresses, GFP-expressing cells begin to make neurons. The FoxD3:GFP-expressing population peaks at approximately 36hpf, which is the developmental stage when the rate of neurogenesis in the hindbrain is at its greatest (Lyons et al., 2003). After 32hpf this cell population does not undergo any great amount of cell division, with BrdU-incorporation and timelapse analysis showing that the majority of GFP-expressing cells are no longer in the cell cycle. The FoxD3:GFP-expressing population then declines rapidly as its cells differentiate into neurons, and some die and are extruded from the neuroepithelium. By 72hpf, the population has dropped sharply and continues to do so, so that by 14dpf, only 9% of their original number at 48hpf remains.

The FoxD3:GFP-expressing population was not analysed in great detail at early stages of development, between 24hpf and 32hpf. Therefore we do not know how the cells of the FoxD3:GFP population divide to expand their number nearly fourfold during this time, however it is likely that they rely on the same mechanisms of largely symmetric division described for other hindbrain progenitors during this period of development (Lyons et al., 2003) . Beyond 32hpf most of these cells do not divide and those that do probably undergo a terminal neuron-pair division. A small number of the FoxD3:GFP-expressing cells become 'mature' radial glia at around 7dpf, but the precise lineage of these cells remains unknown.

Closing Remarks

Here I have presented data that concludes my study of radial glia and neurogenesis in the embryonic zebrafish hindbrain. The Tg(zFoxD3:GFP) stable transgenic line has provided a unique system to observe the same cells that I had characterised in Chapter Three and fate-mapped at the single cell level in Chapter Four. I was able to observe a group of these cells, in the posterior hindbrain, without any form of intervention or manipulation, in an intact embryo, and make observations of how these cells behave as a population, rather than just as single cells. Using timelapse microscopy to monitor these cells during development, I was able to analyse such phenomena as radial migration, cell division and retrograde migration in the *in vivo* situation. Analysis of these timelapse videos corroborates the data from the previous two chapters such as the paucity of cell divisions, the lack of detectable stellate astrocytes and the radial migration of newborn neurons from VZ to more ventral positions. Novel observations of the dynamics of radial migration, such as the interaction between migrating cells and the surrounding radial processes, and also retrograde migration, were made that have never previously been described *in vivo*. The identification of a second, larval population of 'mature' radial glia, derived from a minority of the embryonic GFP-expressing cells, is also a novel observation of interest. I have designated these cells 'mature radial glia' as they resemble the postnatal, differentiated radial glia common to adult fishes, reptiles and amphibians that may perform the role of stellate astrocytes, a cell type that is generally scarce in these vertebrate classes.

There remains much scope for future studies in the Tg(zFoxD3:GFP) transgenic line, such as functional studies that investigate the dependence of migrating cells on the radial processes for correct radial migration, genes that affect radial migration, and factors that control the balance between the populations of the ventricular zone and the mantle layer. Another avenue of research would be, at larval stages, assessing the potential role of GFP-expressing mature radial glia in CNS injury and regeneration.

Chapter Six

General Discussion

The aim of this thesis was to examine and characterise hindbrain neurogenesis during late embryonic zebrafish development and to investigate the role of radial glia in this process. Despite its topical nature the majority of current radial glial research is concentrated on the mammalian cortex, with other brain regions, let alone other organisms, comparatively neglected. Therefore this project would serve an additional purpose in widening the scope of neurogenic radial glial research beyond the mammalian forebrain, and by taking advantage of the external fertilisation, rapid development, fecundity and translucence of the zebrafish I could examine several very fundamental problems that would not be possible to address in mammalian model organisms.

Summary of Results

In Chapter Three, entitled "Characterisation of Ventricular Zone Progenitors during Hindbrain Neurogenesis" I identified a population of mitotically active progenitors with a radial morphology in the ventricular zone (VZ) of the hindbrain during late neurogenesis. These cells were also found to express the intermediate filament GFAP and bore many of the hallmarks of radial glia recently described in the literature (Tamamaki et al., 2001, Noctor et al., 2001, 2002, Miyata et al., 2002, Malatesta et al., 2003 and Anthony et al., 2004). By using an antibody raised to goldfish GFAP I found that most, if not all, progenitor cells in the zebrafish hindbrain VZ express GFAP from approximately 36hpf to 48hpf, an observation not made in other systems: the expression of GFAP in the forebrain of macaque, for example, shows a far higher degree of heterogeneity among progenitor cells (Levitt and Rakic, 1980). However, the timing of expression of GFAP strongly correlates with the neurogenic period of the hindbrain, similar to that observed in the primate cortex (Levitt et al., 1981). The progenitors that remain in the hindbrain after 48hpf downregulate the expression of GFAP and by 72hpf immunoreactivity in the hindbrain is negligible. However, GFAP expression appears to be biphasic in this brain region. By following the embryos for a further 48 hours a small subpopulation of GFAP-expressing cells in the larval VZ becomes recognisable at approximately 5 days post-fertilisation (dpf) by their radial morphology, ovoid cell

somata in the VZ, multiple endfeet that contact the pial surface of the brain and processes that branch extensively on reaching the white matter of the marginal zone. These cells appeared to be a form of differentiated radial astrocyte, to which similar cells have been described in other anamniotes (Miller and Liuzzi, 1986, Bodega et al., 1993, Stensaas and Stensaas, 1968a and Ahboucha et al., 2003). Using a stable transgenic line I visualised the hindbrain VZ during development to show its temporal decline in size and the corresponding expansion of the mantle zone (MZ). In addition, the VZ persists beyond embryonic development and does not appear to be replaced with a sub-ventricular zone (SVZ) as takes place in the mammalian cortex. The cells I have classified as 'mature' or 'glycogen-rich' radial glia lie within this larval VZ, and these cells first appear between the fourth and fifth day of development. These mature radial astrocytes are born after embryonic neurogenesis is complete, and may be the equivalent of mammalian stellate astrocytes (see Miller and Liuzzi, 1986). In mammals, radial glia are generally thought to differentiate into stellate astrocytes *perinatum* (Choi and Lapham 1978, Schmechel and Rakic, 1979, Choi, 1981, Voigt, 1989, Gaiano et al., 2000 and Schmid et al., 2003) and are not found in the adult animal. Here in the zebrafish, I demonstrated that astrocytic radial glia persist long after embryonic development is completed, and the stellate astrocytic differentiation event was not observed.

In Chapter Four, entitled "Lineage Analysis of Late Hindbrain Neural Progenitors" I utilised the technique of single cell intracellular dye injection to fate map the cells of the hindbrain VZ late in development (see Clarke, 1999 and Lyons et al., 2003). Here I showed that over a 24-hour period, ending with zebrafish hatching, the majority of progenitors within the hindbrain VZ either differentiate directly into neurons without dividing, or remain in a state of comparative quiescence. Direct differentiation into neurons, without division, over the timecourse of several cell cycles has been previously reported in lineage analysis of zebrafish hindbrain neurons (Lyons et al., 2003). Two forms of radial migration were identified at the single cell level, one in which cells maintain a bipolar morphology but whose somata enter the mantle layer, and another where the cell has lost its apical process and contact with the ventricular surface and migrates with a unipolar cell shape. The former mode is similar to radial migration that has been described in mouse cortical slice culture as 'somal translocation' (Nadarajah et

al., 2001). Given the respective frequencies observed of both modes of migration, somal translocation is probably a prelude to the latter. Migration with a unipolar morphology has also been reported *in vitro* with the migrating cell described as a 'radial neuron' (Miyata et al., 2001). Here I observed unipolar cells moving to more ventral positions, whilst retaining contact with the marginal zone. Cell division was found to be a rare occurrence and when compared to the huge numbers of cells in the mammalian forebrain, the small size and simplicity of the zebrafish hindbrain makes this result unsurprising. The cell divisions that were observed were found to produce, without exception, two daughter neurons i.e., they were neuron-pair terminal mitoses. Previous lineage analysis has shown this mode of division to predominate in early zebrafish hindbrain neurogenesis (Lyons et al., 2003). In direct apposition to the previously published results of *in vitro* models of mouse cortical development (Miyata et al., 2001 and Noctor et al., 2001 and 2002), asymmetric cell divisions resulting in one neuron and one progenitor, were never observed in the zebrafish hindbrain in my study. Although it has been shown that such divisions do take place in the zebrafish hindbrain, they are a minority (21%) of neurogenic divisions over the course of embryonic development (Lyons et al., 2003).

Since I found that my dye injection protocol only gave good visualisation for 24 hours, I also used a technique first described by Haas and colleagues (Haas et al., 2001) to electroporate single cells with a plasmid encoding for GFP under the control of the α-tubulin or EF1 promoter (Köster and Fraser, 2001), which made it possible to follow the developmental fate of cells for up to seven days. By following the fate of electroporated single progenitor cells for a week, instead of one day as in the dye injection experiment, I showed that long after embryonic development is complete neurogenesis still takes place, with progenitors differentiating into neurons without cell division between one and two days after hatching. From this electroporation study, the cells that I described as 'quiescent' in my dye injection experiment are most likely progenitors that will eventually differentiate into neurons: a number of these GFP-expressing cells were shown to have a quiescent progenitor phenotype i.e., retain contact with the ventricle 24 hours post-electroporation, and then subsequently develop into neurons. Such cells have been previously referred to in the chicken retina as 'nascent' neurons (Henrique et al., 1997). These are cells that have been selected by lateral inhibition to become neurons,

express Delta and NeuroD but have yet to migrate to their appropriate apicobasal position, do not yet express differentiated neuronal markers such as tubulin and do not possess the complete morphology appropriate for their neuronal type. In other words, these are neurons in the process of differentiation; this process may require a comparatively long period of time. In the cat retina, differentiation of horizontal cells is complete one week after migration to the outer plexiform layer, and in total three weeks after these cells are born (Zimmerman et al., 1988). Similarly it has been shown by EM studies that *in vivo*, maturation of astrocytes in the rat optic nerve is a process that requires two to three weeks from when they are born to the completion of their differentiation (Skoff et al., 1976). Therefore it could be that the quiescent cells in my dye injection study are neuronal progenitors that are undergoing differentiation into neurons, but the time required for this differentiation was outside the 24-hour window of the experiment. The alternative explanation is that these quiescent cells are a form of resting, slowly-cycling progenitors that may have the potential to divide or differentiate, but positional information, intrinsic factors or signalling molecules may be retaining these cells in their default state of quiescence.

With respect to the results of the Chapter Five, the 'mature' or 'glycogen-rich' radial glial or astrocytic cells apparent at 5dpf are derived from a subset of these slowly differentiating progenitor cells, but the ultimate fate of the vast majority of the cells in the postembryonic, larval VZ is neuronal. No progenitors were found to give rise to stellate astrocytes, either by dye injection or electroporation, concurring with the majority of previously published data that indicate such astrocytes to be a rare, if not absent, cell type in the teleost brain outside the optic nerve (Nona et al., 1985, Levine, 1989 and Kálmán, 1998). Since I was labelling cells in the dorsal half of the VZ the absence of oligodendrocytes from my study is also not unsurprising since this cell type is derived from the medioventral VZ as I described in Chapter Three. This area is deep within the tissue of the hindbrain and not readily accessible for lineage tracing techniques, so I confined my study to the dorsal VZ. Also, in the zebrafish hindbrain oligodendrocytes tend to remain in very ventral positions even during myelination (Brösamle and Halpern, 2002) making the chance of their being labelled in my experiments even less likely.

In Chapter Five, "Analysis of Hindbrain Neurogenesis in the Stable Transgenic Tg(zFoxD3:GFP) Line" I took advantage of a transgenic line raised in the laboratory of Christiane Nüsslein-Volhard (Gilmour et al, 2002) in which a subset of radial progenitors in the hindbrain VZ express GFP. I analysed these FoxD3:GFP-expressing cells as neurogenesis and embryonic development progressed by using timelapse video microscopy. I was able to observe many of the cellular behaviours revealed in my single cell dye injection experiments, but at the population level rather than as single cells. To my knowledge these videos are the first to show radial migration in real time, in the *in vivo* situation. Migrating FoxD3:GFP-expressing cells also displayed the saltatory motion of migrating neurons as first described *in vitro* by Edmondson and Hatten, showing that this is a real phenomenon that takes place in the intact brain (Edmondson and Hatten, 1987, Gasser and Hatten, 1990a and 1990b). In corroboration with my lineage studies, my timelapse videos showed that cell division was a rare phenomenon at this stage, with only four mitoses observed in six videos each approximately 12 hours in length. However, unlike my fate mapping studies, I was able to observe the cell divisions in real-time, and they were all of the so-called 'parallel' or 'horizontal' nature i.e., parallel to the plane of the ventricular surface, and that none of the divisions were 'perpendicular' such as those that have been described in *in vitro* studies of the rat retina and ferret cortex (Chenn and McConnell, 1995 and Cayouette and Raff, 2003). This observation corroborates well with previous analyses of hindbrain neurogenesis in the zebrafish (Lyons et al, 2003). The persistent expression of GFP up to at least 14dpf in a subset of these radial progenitors of the hindbrain also strengthens my case for identifying 'mature' or 'glycogen-rich' radial glia as described in Chapter Three. By the fourth or fifth day of development these cells were observed with ovoid cell bodies in the dorsal VZ and a long branching pial process with enlarged endfeet contacting the pial surface, readily discernible by strong GFP expression. Some of these cells, and also other cells not expressing the FoxD3:GFP transgene, die at this stage and are extruded from the neural tube into the 4^{th} ventricle. This may be a selective culling of certain cells in the hindbrain, possibly by apoptosis.

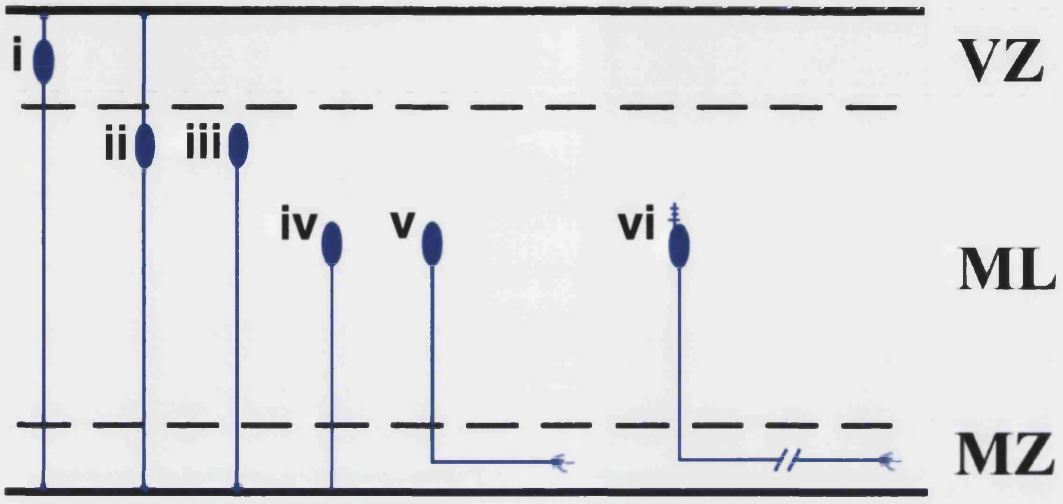
In this thesis I have characterised late neurogenesis in the embryonic zebrafish hindbrain, using a combination of approaches including marker expression, timelapse microscopy analysis of *in vivo* development using transgenics and fatemapping at the level of the single cell. I have demonstrated that an embryonic population of neuronal progenitors possess many of the characteristics of radial glia, such as GFAP expression, radial bipolar morphology and processes acting as a substrate for neuronal migration. They are similar in many regards to the radial glia described in mammalian embryonic systems recently; they are proliferative, neurogenic, populate the ventricular zone almost entirely and their radial processes guide the radial migration of newborn neurons in the mantle layer. However, their mode of cell division differs from mammals, as does their ultimate fate. Later on in development I identified a second population of specialised cells in the hindbrain that also displays radial glial-like characteristics, and these cells persist well beyond embryonic life. This second population is distinct from the earlier in that their radial processes are rich in glycogen granules, and possess a very complex branched, secondary morphology. It is my conclusion that it is these later-appearing cells that are the mature or differentiated astrocytic radial glia of zebrafish. These late-occurring radial glial cells do not arise until after embryonic development is complete, and do not appear to possess the proliferative neurogenic behaviour of the cells known as radial glia in the mouse, nor do they appear to give rise to stellate astrocytes. Their morphology and persistence into postembryonic life is characteristic of radial glia described in many other non-mammalian vertebrates such as amphibians (Zamora, 1978, Miller and Liuzzi, 1986 and Bodega et al., 1993) and reptiles (Stensaas and Stensaas, 1968a and Ahboucha et al., 2003).

A Model of Zebrafish Neurogenesis

Many studies have hypothesised the sequence of events when a single progenitor transforms into a neuron, but none has yet described this transformation in zebrafish. Using evidence accrued from antibody staining, single-cell labelling and timelapse movies I present an inferred model of this process in the zebrafish hindbrain (Figure 6.1). After 48hpf, the vast majority of neurons in the zebrafish hindbrain are derived from direct differentiation, rather than from a cell division (see Chapter Four). The first event is a rapid somal translocation of the cell body from VZ into the MZ, all the while

maintaining apical and basal processes. This differs from mouse somal translocation, since in the mouse it has been described that often cytoplasmic contact is lost with the ventricle and instead there is a shortened trailing process (Nadarajah et al., 2001). The ventricular contact is then lost, although I cannot be sure whether the ventricular process collapses, or is retracted by the soma as occurs in marsupials (Morest, 1970). The pial process terminates within the marginal zone, and sometimes ends in an endfoot or enlarged club in contact with the pial surface. This generates a monopolar cell with its cell body residing in the mantle zone. At this point the cell most likely expresses the neuronal marker HuC, and its soma may adopt a rounded or polygonal shape. Following this change to monopolar morphology, the cell body may migrate further to more ventral positions in the MZ; this may be a fine-tuning or cell sorting event, to ensure the cell is in its correct final position. During this time, or possibly shortly afterwards, the basal process of the newborn neuron directly transforms into an axon, which grows in the direction appropriate for its neuronal type. The extension and growth of the neuronal axon precedes the development of dendritic elaborations on the soma. These dendrites appear between three to four days after the initial expression of HuC protein. With the development of dendrites the neuron loses its transient monopolar form and begins to adopt its final, differentiated neuronal morphology.

Figure 6.1. Proposed mechanics of neuronal differentiation in zebrafish hindbrain
A cartoon showing a model of progenitor transformation into a neuron. This schematic has been constructed from observations and data from all three results chapters. Ventricular zone (VZ), mantle layer (ML) and marginal zone (MZ) are indicated by solid or broken lines.
The initial morphology of the radial progenitor is bipolar, with soma lying in the VZ and processes, terminating in endfeet, contacting both ventricular and pial surfaces (i). Following the decision to differentiate, a rapid translocation of the soma from VZ to ML takes place, ii, whilst maintaining both processes. The cell then loses its ventricular process, possibly by retracting it into its soma (iii). Subsequently, the cell migrates toward the pia, this time by a different mode of locomotion, apparently shortening its basal process. This basal process is then recycled by the newborn neuron (v) and transforms directly into an axon. After the initial axonal outgrowth, the soma begins to develop dendritic elaborations (vi) and soon adopts the final morphology of a differentiated neuron.


i
ii
iii
iv
v
vi
VZ
ML
MZ

Are Radial Glia, Radial Glia?

The transformation of radial glia from transient scaffolding cells to interesting, proliferative, neural stem cell-like cell has stimulated many studies in laboratories worldwide and with it, the inevitable differing conclusions and opinions. Even just focussing on the developing mouse cortex several laboratories have interpreted their data in different ways and there remain many issues that have yet to be resolved and provide much scope for discussion over the coming years. It is interesting to read in the literature that as long ago as when radial glia were first described in the late 19th century there was considerable confusion and disagreement in the field and even the ever-eminent Santiago Ramón y Cajal, who laid the foundation for so much of our understanding of developmental neurobiology, seemed uncertain as to the true nature of radial glia (see Bentivoglio and Mazzarello, 1999 and Rakic, 2003, and references within). It is ironic that now, over a century later with all the tools of modern science at our fingertips, we are asking the same questions as then: are radial glia a separate lineage from neurons? Is there a difference between a radial glial progenitor and a neuronal progenitor? Is there even such a thing as a radial glial cell?

Radial glia were first identified in the late Victorian era, as a stain-incorporating cell that became distinct in the ventricular zone (VZ) when slices of foetal mammalian forebrain were stained by the metal impregnation technique invented by Camillo Golgi. Guiseppe Magini, the Italian histologist who should be credited with their discovery, described such cells as 'radial nevroglia' (Magini 1888, see Bentivoglio and Mazzarello, 1999), presumably because he could not decide whether the cells were neurons or glia. However, it is now known in modern times that the dense precipitate of the Golgi stain labels in a stochastic fashion; according to Rakic, who has apparently quantified the labelling frequency of the stain, between 1% and 3% of cells in any Golgi-stained section become impregnated with the label (Rakic, 2003). Rather than the diffuse, regular network of radial processes as shown in some reports (Rakic, 1972) in reality the radial cells composing the VZ are packed together tightly and their processes less ordered; an analogy would be the comparison between a planted orchard and a dense forest of trees. Therefore early researchers may have been misled by Golgi-stained preparations of cortex, believing the stain had been incorporated into a specific cell type within the VZ, instead of labelling all manner of radial cells such as progenitor cells,

radial glia, nascent neurons and transitional forms. Therefore the Golgi stain alone is not a good criterion for the identification of radial glia, although this was how such cells were first described, in the late 19th century (see Bentivoglio and Mazzarello, 1999, and Jacobson, 1991 and references therein). In recent times however, despite the efforts of many researchers to find a specific marker of radial glia, the result has been greater confusion. Mammalian cells described as radial glia share morphology (Morest and Silver, 2003), a variety of marker expression including RC1, transitin, RC2 and nestin (Hartfuss et al., 2001, Götz et al., 2002 and Malatesta et al., 2003), ventricular-projecting cilium (Tramontin et al., 2003) and even intracellular electrophysiological characteristics (Bittman et al, 1997, Noctor et al, 2004) with neuroepithelial, undifferentiated radial progenitor cells. The original marker for radial glia described by Rakic is the intermediate filament GFAP (Levitt and Rakic, 1980), although this protein is not expressed in the radial glia of rodents (Bignami and Dahl, 1974), which today probably comprise the most common vertebrate model systems for developmental neurobiology experiments. As the mammalian radial glial story unravels, opinion in the field appears to be divided; those who support the theory of radial glia as a separate differentiated cell type, a subtype of neuroepithelial cell (see reviews by Campbell and Götz, 2002 and Tramontin et al., 2003), and those who feel that the term is outdated by our current knowledge of neurogenesis, and that there is no difference between the cells we call neuroepithelial cells and those we call radial glia (see reviews by Parnavelas and Nadarajah, 2001, Fishell and Kriegstein, 2003 and Morest and Silver, 2003). Experimental evidence exists for both cases. Electrophysiological recordings taken from cortical VZ radial glial cells showed no difference with progenitor cells (Bittman et al., 1997 and Noctor et al, 2004) and Noctor and colleagues report that most, if not all, the cells in the cortical VZ are radial glia (Noctor et al., 2001, 2002 and 2004). However, immunostaining to markers of radial glia such as BLBP, RC2 and GLAST indicate that there is heterogeneity in the radial glia population (Hartfuss et al., 2001 and Malatesta et al., 2003). Hartfuss and colleagues showed that at E14, when neurogenesis is at its peak, 12% of cortical progenitors are immunonegative for RC2, BLBP and nestin, yet express the Ki-67 antigen (Hartfuss et al., 2001). Therefore the question remains whether or not there are any undifferentiated neuroepithelial cells, but with the same morphology as radial glia, during mid- and late embryonic cortical development, as the Hartfuss study

implies, or if all the cells in the VZ during the period of neurogenesis are radial glia, as Noctor and colleagues suggest. In this study, comparisons with the mouse situation are hard to make since GFAP expression appeared to be much more homogeneous, with all the progenitors of the hindbrain VZ expressing the intermediate filament.

Zebrafish Radial Glia

My own definition of radial glia cannot be formed without giving careful consideration to previously published reports of radial glia. However, since my model organism is a teleost fish, differences across vertebrate taxa must also be considered, especially the astroglial repertoire of anamniotes that differs in several respects to that of mammals and birds. In my studies, the cells that comprise the embryonic VZ during late neurogenesis possess morphological characteristics that would indicate radial glial phenotype: they express GFAP, a marker of radial glia and astrocytes, and they possess a slim or ovoid cell soma and radial, bipolar cell morphology, with processes contacting both ventricular and pial endfeet. However, since almost all VZ cells possess a radial morphology and due to the transient nature of many markers in progenitors, molecular marker expression and cell morphology may not be good criteria to distinguish radial glia. Therefore the functional characteristics of these embryonic radial glial cells should also be considered. Much like the radial glia of mammals, the radial VZ cells identified in my studies are neurogenic, and their basal processes are substrates for the radial migration of newborn neurons. To compare these observations, ticked features are those mammalian and zebrafish radial glia share, and those crossed are the differences in behaviour displayed by the radial glia of the fish:

- ✓ Express GFAP
- ✓ Radial, bipolar morphology
- ✓ Neurogenic
- ✓ Processes are a substrate for radial migration
- × Neurogenic by differentiation
- × Symmetric cell divisions only
- × Ultimate fate is either neuronal or occasional radial astrocyte; no stellate astrocytic fate

By this list, both the physical characteristics of GFAP expression with radial, bipolar morphology and the functional, i.e., contribution to neurogenesis and radial processes as a substrate for radial migration weigh heavily toward arguing that these embryonic, radial VZ cells are indeed zebrafish embryonic radial glia. The three 'cons' are all explainable by phylogenetic differences between bony fishes and mammals. Zebrafish radial glia are neurogenic by direct differentiation, instead of highly proliferative by asymmetric divisions, because the teleost hindbrain has a much smaller requirement of neurons, but in a shorter period of neurogenesis (see Discussion in Chapter Four). Differentiation into stellate astrocytes was not observed in zebrafish radial glia in my study, but this is because there are probbaly no stellate astrocytes in the CNS of teleosts (Nona et al., 1985 and Levine, 1989; see Kálmán, 1998), outside the optic nerve. However, it is likely the 'radial astrocytes' that are observed both by GFAP expression and FoxD3:GFP expression during larval development are the anamniotes' version of stellate astrocytes (see Miller and Luizzi, 1986). Therefore it is possible to call the radial progenitors in the zebrafish hindbrain between 24hpf and 72hpf the equivalent of mammalian radial glia, given the properties shared between the two, but also taking into consideration the evolutionary differences between teleost fishes and mammals.

Fish Are Not Mammals Or, Evolutionary and Comparative Perspectives

In order to be considered 'of use' the aim of much research is to demonstrate parallels with human biology, therefore there may be people who decry the use of fish for novel, informative brain research and consider only the mouse or rat as a 'proper' model system. Considering the data from all three chapters together, it is clear that there is considerable difference in many aspects of neurogenesis in the zebrafish when compared to previously published research of mammalian systems. However, much of my data is not comparable to mammalian work simply because analysis of this kind is not possible in the mouse, for example, the live imaging, single-cell dye injection, single-cell electroporation and timelapse videos in my project. Therefore work such as I have presented here must be considered useful, since it is not possible to perform such studies in a mammalian model system.

The zebrafish is a comparatively new model organism, and certain basic characterisations are still in their early stages, such as neuronal subtype classification, zebrafish-specific antibodies and neuroanatomy. One aspect I regret not examining in greater detail is the histology of the zebrafish hindbrain, especially at the level of the electron microscope, during larval development. It is not known for example, if there is a subventricular zone (SVZ) in the hindbrain, or if and when an ependymal layer develops at the dorsalmost extreme of the VZ. The GFP-negative domain in the Tg(HuC:GFP) transgenic would be a good system to examine by electron microscopy to look for such cells as ciliated ependymoglia, tanycytes with radial processes or any kind of stellate cell in this area. Similarly, the characterisation of neurons in the hindbrain would add an extra dimension to my studies; although there are well over 2000 neurons in each rhombomere by 48hpf (Lyons et al., 2003), the function, structure and projections are only known for a minority of this number, such as reticulospinal neurons (Metcalfe et al., 1986 and Gahtan and O'Malley, 2003). An electroporation survey in the Tg(HuC:GFP) transgenic using DS-Red or a similar RFP would reveal much information about axon projections and neuronal circuitry in the hindbrain. Being able to discern different types of neuron may reveal asymmetry in neuronal fate in my lineage studies, especially in the instances of the neuron-pair divisions observed in my dye injection experiment. While outwardly symmetric by my method of phenotyping, i.e., by HuC:GFP expression, the cell divisions may be asymmetric in that they produce two different types of neuron.

Early descriptions of stellate astrocytes indicated that they were a cell type specific to birds and mammals, having evolved due to the greater complexity of the brains of these animals (see Ramón y Cajal, 1911). However, Ramón y Cajal's hypothesis is probably an oversimplification, as it has subsequently been shown that the development of such astrocytes is not exclusively associated with higher phylogeny. Stellate astrocytic cells have been demonstrated in chondroichthyeans such as *Torpedo* and *Raia* and also in agnathans; according to Wasowicz and colleagues, Gustaf Retzius apparently identified stellate astrocytes in *Petromyzon*, and over a hundred years later Wicht and colleagues used an antibody to GFAP to label these cells (Retzius, 1893, Kálmán and Gould, 2001, Wasowicz et al., 1999 and Wicht et al., 1994). However, the results of Wicht and

colleagues remain contentious since other researchers have been unable to demonstrate stellate astrocytes in agnathans by the same methods (Dahl and Bignami, 1973). Among jawed fishes too the story is unclear, since some chondrostei like *Raia* appear to possess a form of stellate astrocyte whilst others such as the spiny dogfish *Squalus* do not (Kálmán and Gould, 2001) whereas in the brains of teleosts, stellate astrocytes are only ever found in the optic nerve (Nona et al., 1985, Levine, 1989 and Kálmán, 1998). Horstmann hypothesises that stellate astrocytes are derived in response to an increasing thickness and complexity of the brain (Horstmann, 1954). In support of this, by visualising these cells by GFAP antibody staining in various cartilaginous fish, Kálmán states that the evolution of these cells in the chondrostei has been an event parallel to amniotes (Kálmán and Gould, 2001 and Kálmán and Ari, 2002). Again, much like our knowledge of radial glia, in the case of stellate astrocytes too, our theories of glial phylogeny in vertebrates are proving to be an oversimplified model of the reality.

The exciting field of neural stem cells is progressing rapidly. One very interesting avenue that was not explored in the course of my studies was the potential for radial glial-mediated regeneration following CNS injury. The late-occurring radial glia identified in my study may appear to be postmitotic, but could also be in a quiescent state and could possibly be stimulated to re-enter the cell cycle, such as by mechanical or chemical insult to the brain. One interesting idea proposed after investigations of amphibian spinal cord astroglia is that with the absence of stellate astrocytes, astrocytic radial glial cells may be performing the various roles that these cells do in mammals (Zamora, 1978, Miller and Liuzzi, 1986 and Bodega et al., 1990). Whether or not this hypothesis can be extended to all anamniotes is untested, but given the lack of demonstrable stellate astrocytes in teleost fish (outside the optic nerve) this concept is not entirely fantastic. It has been shown in the adult mouse forebrain that quiescent SVZ astrocytes can replace transit amplifying cells – that normally give rise to olfactory bulb neurons – that have been chemically ablated (Doetsch et al., 1999, Tramontin et al., 2003). Therefore the yet-untested possibility remains that the post-embryonic, larval radial glia that I identified in my studies may possess the neural stem cell qualities of mammalian quiescent, stem cell-like astrocytes as described by Doetsch and colleagues. A wounding assay using either mechanical injury or laser ablation of CNS tissue and

observing the subsequent behaviour of GFP-expressing radial glia in Tg(zFoxD3:GFP) larvae would be an easy way to begin addressing this question.

Neurogenesis Without Asymmetric Cell Divisions

There is very little cell division of progenitors in the hindbrain after 48hpf. The few divisions that were observed in my lineage study were all symmetric, neuron-pair terminal divisions. So the question remains: what is the source of these late progenitors that differentiate into neurons – and to a lesser extent, mature radial glia – after 48hpf without dividing? Asymmetric cell division is considered to be key in generating large numbers of differentiated cells whilst maintaining a constant progenitor pool. Therefore the absence of these divisions is puzzling, considering the widely held belief that such a mode of division is characteristic of the developing CNS in most vertebrate systems (reviewed by Wodarz and Huttner, 2003). However, *in vivo* lineage analysis in the early zebrafish hindbrain has shown this not to be the case in the fish: Lyons and colleagues describe how 84% of hindbrain neurons born between neural keel stage and 48hpf are derived from neuron-pair symmetric divisions (Lyons et al., 2003). The same study also demonstrated that less than 10% of the original hindbrain progenitor population remains by 48hpf, so these cells are in some way being restrained or reserved for the neurogenesis that takes place after 48hpf, and also to a lesser extent, for the generation of mature radial glia that occurs between 4dpf and 5dpf. The stage at which these cells become earmarked or selected for this late differentiation is not known, although from my data, at 48hpf over 95% of FoxD3:GFP-expressing cells no longer incorporate BrdU, and may have exited the cell cycle before this stage.

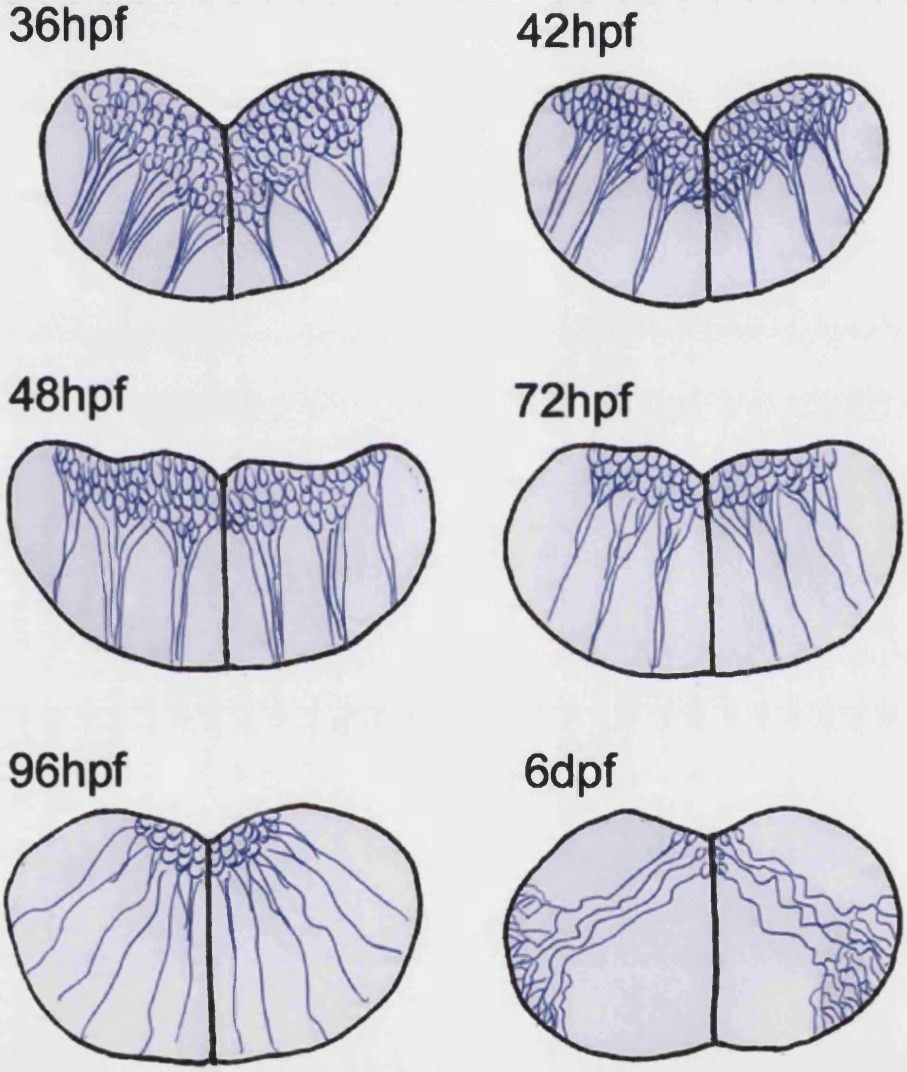
Unlike the mammalian cortex, where it is believed that the VZ is largely maintained – by asymmetric cell divisions – in size through the majority of neurogenesis (see Takahashi et al., 1996 and Cai et al., 2002) the zebrafish hindbrain appears to be different. After 36hpf the VZ becomes steadily depleted as developmental age increases through to hatching (see Chapter Three, Figure 3.3 and Chapter Four, Figure 4.1). This progressive depletion of the progenitors of the VZ is schematically represented in Figure 6.2. Groups of VZ progenitors progressively differentiate into neurons, possibly in cohorts or waves. These neurons migrate radially along the processes that form the glial

curtains to reach their appropriate position within the MZ. This process takes place at its highest rate between 36hpf and 48hpf. This neurogenesis continues and the VZ is continually depleted until 72hpf. There is also a period of cell death in the VZ that occurs from approximately 72hpf through to 7dpf. This cell death further depletes the VZ during early larval life. It is not known if this is a developmental process to remove young neurons that have failed to make appropriate synaptic connections, or is a deliberate culling of progenitors that may be in excess, although the presence of dead cells in the dorsal VZ suggests the latter. However, it is clear that one of the cell types remaining within the postembryonic VZ is the mature radial glia, or radial astrocyte. Whether there are any undifferentiated progenitors co-residing with these astrocytes in the larval VZ is not known, although there is a fairly large proportion of HuC:GFP-negative cells still in the hindbrain by 14dpf.

Division Orientation

The question of division orientation and the correlation with fate continues to stir controversy in vertebrate developmental neurobiology. Recent *in vivo* studies have showed that, contrary to the popular hypothesis of Chenn and McConnell, 'perpendicular' asymmetric cell divisions are a rarity in the developing retina and hindbrain of zebrafish (Chenn and McConnell, 1995, Das et al., 2003 and Lyons et al., 2003). Lyons and colleagues showed that many 'horizontal' cell divisions, deemed symmetric by the McConnell hypothesis, could actually be asymmetric in terms of daughter cell fate. Between 18 and 30hpf, the authors detected only 12 out of 557 cell divisions that occurred out of the plane of the ventricular zone. In my timelapse movies of the developing caudal hindbrain in the Tg(zFoxD3:GFP) transgenic line, all cell divisions observed, though few in number, were also all 'horizontal' in nature. No perpendicular cell division was observed. Whether the lack of perpendicular cell divisions is peculiar to fishes, or are a rarity in all vertebrates *in vivo* remains to be seen. With the advent of new technology and better microscopy, one could hope that the issue of vertebrate division orientation be resolved. However, timelapse studies in brain slices or explant cultures continue to produce opposing results. Several authors have been

Figure 6.2. Depletion of progenitors of the ventricular zone with development
A cartoon of the hindbrain in transverse section. Only progenitor cells in the VZ are illustrated, here in blue.
Radial progenitors of the VZ project their basal processes ventrally into the glial curtains. These cells differentiate directly into neurons and migrate into the mantle layer below. Between 36hpf and 48hpf represents the period where the rate of neurogenesis is at its highest. Since asymmetric cell division is uncommon at this stage, the VZ is not maintained and becomes progressively depleted of progenitors as development continues. From 72hpf until at least 6dpf there is cell death at the dorsal midline, including death of VZ progenitors, further reducing their number. Between 4dpf and 6dpf mature radial glia become evident, which may be a form of progenitor in the adult.


36hpf
42hpf
48hpf
72hpf
96hpf
6dpf

unable to reproduce the perpendicular cell divisions described by Chenn and McConnell: the study of Miyata and colleagues report less than 6% of the divisions they observed at the ventricular surface were vertical or perpendicular, with no correlation with cell fate (Miyata et al., 2001). Tamamaki and colleagues deny their existence altogether, reporting that radial glia divide asymmetrically in the plane of the ventricle (Tamamaki et al., 2001). In contrast to these results, timelapse videos of the *in vitro* culture of newborn rat retina by Cayouette and Raff show similarities with the McConnell model (Cayouette and Raff, 2003). The authors describe 58% of progenitor cell divisions in the plane of the ventricle and 34% perpendicular to it, with the progeny of horizontal cell divisions apparently developing into photoreceptors and those of perpendicular divisions differentiating into cells of differing types. In my opinion, the argument of Huttner and Brand is the most convincing: minute deflections in the angle of vertical cleavage can result in asymmetric determinant inheritance (Hutter and Brand, 1997). However, how this issue will be resolved in mammals is not obvious, since the process cannot be directly observed in the intact brain. Looking to zebrafish for the answer has appeared fruitless, since they do not appear to possess the phenomenon of perpendicular cell divisions in any high proportion (Das et al., 2003 and Lyons et al., 2003). The debate may probably ensue as long as there are studies of vertebrate neurogenesis.

Postnatal Neurogenesis

Nearly forty years ago Altman and Das reported apparent hippocampal neurogenesis in the adult rat (Altman and Das, 1965) although the concept of neurogenesis terminally concluding at perinatal stages is only recently being reviewed. More recently in non-mammalian vertebrates, the work of Arturo Alvarez-Buylla in songbirds and chicken (Alvarez-Bullya and Nottebohm, 1988 and Alvarez-Buylla et al., 1990) and Jose Garcia-Verdugo in reptiles (Garcia-Verdugo et al., 1986) have indicated that adult neurogenesis is a real phenomenon. Mammalian postnatal neurogenesis and the question of adult neural stem cells is a very topical subject now, given the potential rewards of such discoveries. It has since been shown that certain types of glia may hold the key to this process (reviewed by Doetsch, 2003, and Goldman, 2003). Doetsch and colleagues identified SVZ astrocytes as a source of migratory interneurons contributing to the

olfactory bulbs in adult mice, corroborated by other researchers (Doetsch et al., 1999, Capela and Temple, 1999 and Tramontin et al., 2003). One puzzling question that remains to be answered is why the capacity for adult neurogenesis apparently decreases with higher phylogeny. It is well documented in many teleost fish – including cichlids, cyprinids, poecilids and gymnotiformes – that physiological, constituitive neurogenesis takes place in many brain regions throughout adult life such as the eye (Mack and Fernald, 1997 and Marcus et al., 1999) and cerebellum (Kranz and Richter, 1970 and Zupanc, 1999). Possibly mediated by the same mechanisms of adult neurogenesis, regeneration of large areas of brain tissue has also been demonstrated in fishes (Zupanc and Clint, 2001 and 2003). In contrast, mammals do not show such capabilities; for example, the neurons lost in injury such as ischaemia or neurodegenerative disorders in humans – such as neurons of the nigrostriatal pathway lost in Parkinson's Disease – are not replaced. Therefore in such diseases in humans, adult neurogenesis in the brain does not respond to pathological loss of brain cells. One current hypothesis is that the more advanced brains of higher vertebrates lack certain permissive factors that allow neurogenesis to take place (see Zupanc, 1999). The only evidence for this, however, is that certain cells isolated from adult mammalian brain tissue and grown *in vitro* can be induced to behave like multipotent, self-renewing stem cells by manipulating their culture conditions. In my studies I have demonstrated that in the zebrafish neurogenesis takes place, at a low level, in the hindbrain beyond embryonic development and into larval life. According to Günther Zupanc, it is teleost fish that display the highest level of adult neurogenesis of all vertebrates (Zupanc, 1999 and 2001). In my thesis I did not examine neurogenesis beyond early larval stages, but there is clearly much scope for future work following my studies, in the adult zebrafish on the exciting subject of adult neurogenesis and regeneration.

Ending Neurogenesis

The controlled termination of neurogenesis must surely be as important as its correct initiation. How this happens in the zebrafish is not clear; neurogenesis may be curbed or reigned in during late embryonic and early larval life in a number of ways. The *in vivo* lineage analysis of zebrafish hindbrain neurogenesis by Lyons and colleagues showed

that by 48hpf, 92% of lineage trees reconstructed from single labelled progenitors terminated in neurons only (Lyons et al., 2003) therefore there appeared to be very few self-propagating or asymmetric progenitor cell divisions. Considering these data, neurogenesis in the hindbrain may be brought to a close by simply running out of progenitors. However, this may not be the complete story. In Chapter Three, my BrdU-incorporation analysis shows that a small number of hindbrain ventricular zone (VZ) cells still incorporate BrdU by 5dpf, demonstrating that there are cells in cycle still remaining at this stage. Furthermore, looking at the Tg(HuC:GFP) hindbrain in transverse section, there remains a prominent HuC:GFP-negative ventricular zone (VZ) at this stage, which persists up to 14dpf. In the literature, there are several reports of neurogenesis throughout adult life in teleost fishes (Mack and Fernald, 1997, Marcus et al., 1999 and Zupanc, 1999). This neurogenesis should not be possible if there were no progenitor cells remaining in the adult CNS.

A second hypothesis is that the progenitors leave the cell cycle not by undergoing terminal differentiation, but becoming by becoming arrested in G1-phase and entering a quiescent, G0-like state. Cell cycle arrest, and sometimes differentiation, can be induced through an accumulation of the cyclin-dependent kinase inhibitor p27(Kip1) (Toyoshima and Hunter, 1994, for review, see Edlund and Jessell, 1999 and Durand and Raff, 2000). Without functional studies, it is not possible to know if this is exactly happening in the zebrafish hindbrain, although the importance of p27(Kip1) in the CNS has been demonstrated. Work in the laboratory of Martin Raff has shown that the accumulation of p27(Kip1) in oligodendrocyte precursor cells (OPCs) determines if the OPC should continue to proliferate, or to leave the cell cycle and terminally differentiate into an oligodendrocyte (Durand et al., 1997 and Gao et al., 1997). From my observations in Chapter Three, there are certainly cells persisting in the hindbrain VZ at 4 to 5dpf that do not incorporate BrdU and do not express HuC:GFP in the Tg(HuC:GFP) transgenic line; whether or not these cells have entered some form of quiescent state is yet to be tested.

Thirdly, a recent study has indicated that apoptosis is an important mechanism for controlling neuroblast proliferation in the abdomen of the postembryonic *Drosophila* (Bello et al., 2003). Bello and colleagues showed that abdominal postembryonic

neuroblasts (pNBs) gave rise to, on average, 5.4 neurons with no clone greater than 13 neurons in size, whilst thoracic pNBs generated clones of approximately fifty neurons in the same timeframe (100 hours after hatching). Thoracic clones were also found to still retain one neuroblast in its number, whilst abdominal clones lost theirs. Abdominal clones with mutations in the proapoptotic genes *reaper*, *grim* and *head involution defective* experienced an increase in mean clone size to almost the same level as thoracic pNB-derived clones, and also retained a single pNB in the clone. This apoptosis that restrains the neurogenesis of abdominal pNBs is dependent upon the homeotic gene *abdominal-A*, or *abdA*: abdominal clones with mutations in *abdA* showed the same phenotype as the mutants in proapoptotic genes. The homeotic gene *abdA* is expressed in the central abdomen but not the thorax during development.

Although I did not perform any functional studies at all to be able to make a direct comparison with the *Drosophila* situation, there does exist a modicum of circumstantial evidence. Some FoxD3:GFP-expressing cells, as well as cells not expressing the transgene, die and are extruded from the neural tube from 72hpf, the timing coinciding with the end of embryogenesis and also concurrent with the sharp decline in neurogenesis that takes place in the hindbrain at this stage (Lyons et al., 2003). However, whether this cell death is apoptosis was not tested, but it is an interesting hypothesis to contemplate.

Future Directions

Although I have presented much new *in vivo* data here of observations of radial glia and neurogenesis, the genetic and environmental control of these processes, especially late in development, remains comparatively unexamined. As is usually the case with such investigations, the results of my research have further produced a number of new questions. For example, the stem cell-like properties of the late-occurring, mature radial glia I identified have yet to be investigated. The zebrafish undergoes a metamorphosis-like event at approximately two weeks post-fertilisation; the role of these cells and neurogenesis at this time is also unknown. Given the recent reports of Notch signalling in stem cell-like cells of the glial lineage, my model system here would provide excellent opportunity for *in vivo* genetic manipulations and functional studies of these

glial cells, and their involvement in adult or regenerative neurogenesis. The zebrafish is a fairly new subject for developmental studies, and it is my hope that this characterisation has contributed at least partly to our knowledge of the development of what is rapidly becoming a very exciting model system.

References

Ahboucha S, Laalaoui A, Didier-Bazes M, Montange M, Cooper HM, Gamrani H (2003) Differential patterns of glial fibrillary acidic protein-immunolabeling in the brain of adult lizards. *J Comp Neurol* 464:159-171.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol* 124:319-335.

Alvarez-Buylla A, Buskirk DR, Nottebohm F (1987) Monoclonal antibody reveals radial glia in adult avian brain. *J Comp Neurol* 264:159-170.

Alvarez-Buylla A, Kirn JR (1997) Birth, migration, incorporation, and death of vocal control neurons in adult songbirds. *J Neurobiol* 33:585-601.

Alvarez-Buylla A, Ling CY, Yu WS (1994) Contribution of neurons born during embryonic, juvenile, and adult life to the brain of adult canaries: regional specificity and delayed birth of neurons in the song-control nuclei. *J Comp Neurol* 347:233-248.

Alvarez-Buylla A, Theelen M, Nottebohm F (1990) Proliferation "hot spots" in adult avian ventricular zone reveal radial cell division. *Neuron* 5:101-109.

Anderson SA, Eisenstat DD, Shi L, Rubenstein JL (1997) Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science* 278:474-476.

Ando R, Hama H, Yamamoto-Hino M, Mizuno H, Miyawaki A (2002) An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc Natl Acad Sci U S A* 99:12651-12656.

Anthony TE, Klein C, Fishell G, Heintz N (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron* 41:881-890.

Arochena M, Anadon R, Diaz-Regueira SM (2004) Development of vimentin and glial fibrillary acidic protein immunoreactivities in the brain of gray mullet (Chelon labrosus), an advanced teleost. *J Comp Neurol* 469:413-436.

Bao ZZ, Cepko CL (1997) The expression and function of Notch pathway genes in the developing rat eye. *J Neurosci* 17:1425-1434.

Bartsch S, Bartsch U, Dorries U, Faissner A, Weller A, Ekblom P, Schachner M (1992) Expression of tenascin in the developing and adult cerebellar cortex. *J Neurosci* 12:736-749.

Bello BC, Hirth F, Gould AP (2003) A pulse of the Drosophila Hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. *Neuron* 37:209-219.

Bentivoglio M, Mazzarello P (1999) The history of radial glia. *Brain Res Bull* 49:305-315.

Berry M, Rogers AW (1965) The migration of neuroblasts in the developing cerebral cortex. *J Anat* 99:691-709.

Bignami A, Dahl D (1974) Astrocyte-specific protein and radial glia in the cerebral cortex of newborn rat. *Nature* 252:55-56.

Bodega G, Suarez I, Fernandez B (1990) Radial astrocytes and ependymocytes in the spinal cord of the adult toad (Bufo bufo L.). An immunohistochemical and ultrastructural study. *Cell Tissue Res* 260:307-314.

Bodega G, Suarez I, Rubio M, Villalba RM, Fernandez B (1993) Astroglial pattern in the spinal cord of the adult barbel (Barbus comiza). *Anat Embryol (Berl)* 187:385-398.

Bovolenta P, Liem RK, Mason CA (1984) Development of cerebellar astroglia: transitions in form and cytoskeletal content. *Dev Biol* 102:248-259.

Brenner M (1994) Structure and transcriptional regulation of the GFAP gene. *Brain Pathol* 4:245-257.

Brosamle C, Halpern ME (2002) Characterization of myelination in the developing zebrafish. *Glia* 39:47-57.

Butler, AB, and Hodos, W (1996). Comparative Vertebrate Neuroanatomy: Evolution and Adaption.

Cai L, Hayes NL, Takahashi T, Caviness VS, Jr., Nowakowski RS (2002) Size distribution of retrovirally marked lineages matches prediction from population measurements of cell cycle behavior. *J Neurosci Res* 69:731-744.

Calver AR, Hall AC, Yu WP, Walsh FS, Heath JK, Betsholtz C, Richardson WD (1998) Oligodendrocyte population dynamics and the role of PDGF in vivo. *Neuron* 20:869-882.

Campos LS, Duarte AJ, Branco T, Henrique D (2001) mDll1 and mDll3 expression in the developing mouse brain: role in the establishment of the early cortex. *J Neurosci Res* 64:590-598.

Capela A, Temple S (2002) LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. *Neuron* 35:865-875.

Caviness VS, Jr., Rakic P (1978) Mechanisms of cortical development: a view from mutations in mice. *Annu Rev Neurosci* 1:297-326.

Cayouette M, Raff M (2003) The orientation of cell division influences cell-fate choice in the developing mammalian retina. *Development* 130:2329-2339.

Cayouette M, Whitmore AV, Jeffery G, Raff M (2001) Asymmetric segregation of Numb in retinal development and the influence of the pigmented epithelium. *J Neurosci* 21:5643-5651.

Chambers CB, Peng Y, Nguyen H, Gaiano N, Fishell G, Nye JS (2001) Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors. *Development* 128:689-702.

Chanas-Sacre G, Rogister B, Moonen G, Leprince P (2000) Radial glia phenotype: origin, regulation, and transdifferentiation. *J Neurosci Res* 61:357-363.

Chenn A, McConnell SK (1995) Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. *Cell* 82:631-641.

Chernoff EA (1996) Spinal cord regeneration: a phenomenon unique to urodeles? *Int J Dev Biol* 40:823-831.

Chetverukhin VK, Polenov AL (1993) Ultrastructural radioautographic analysis of neurogenesis in the hypothalamus of the adult frog, Rana temporaria, with special reference to physiological regeneration of the preoptic nucleus. I. Ventricular zone cell proliferation. *Cell Tissue Res* 271:341-350.

Choi BH (1981) Radial glia of developing human fetal spinal cord: Golgi, immunohistochemical and electron microscopic study. *Brain Res* 227:249-267.

Choi BH, Lapham LW (1978) Radial glia in the human fetal cerebrum: a combined Golgi, immunofluorescent and electron microscopic study. *Brain Res* 148:295-311.

Clarke JD (1999) Using fluorescent dyes for fate mapping, lineage analysis, and axon tracing in the chick embryo. *Methods Mol Biol* 97:319-328.

Clarke JD, Alexander R, Holder N (1988) Regeneration of descending axons in the spinal cord of the axolotl. *Neurosci Lett* 89:1-6.

Clarke JD, Erskine L, Lumsden A (1998) Differential progenitor dispersal and the spatial origin of early neurons can explain the predominance of single-phenotype clones in the chick hindbrain. *Dev Dyn* 212:14-26.

Clarke JD, Lumsden A (1993) Segmental repetition of neuronal phenotype sets in the chick embryo hindbrain. *Development* 118:151-162.

Clint SC, Zupanc GK (2001) Neuronal regeneration in the cerebellum of adult teleost fish, Apteronotus leptorhynchus: guidance of migrating young cells by radial glia. *Brain Res Dev Brain Res* 130:15-23.

Clint SC, Zupanc GK (2002) Up-regulation of vimentin expression during regeneration in the adult fish brain. *Neuroreport* 13:317-320.

Concha ML, Russell C, Regan JC, Tawk M, Sidi S, Gilmour DT, Kapsimali M, Sumoy L, Goldstone K, Amaya E, Kimelman D, Nicolson T, Grunder S, Gomperts M, Clarke JD, Wilson SW (2003) Local tissue interactions across the dorsal midline of the forebrain establish CNS laterality. *Neuron* 39:423-438.

Cowan NJ, Lewis SA, Balcarek JM, Krek V, Shelanski M (1985) Structural implications of a cDNA clone encoding mouse glial fibrillary acidic protein. *Ann N Y Acad Sci* 455:575-582.

Dahl D, Bignami A (1973) Immunochemical and immunofluorescence studies of the glial fibrillary acidic protein in vertebrates. *Brain Res* 61:279-293.

Dahl D, Crosby CJ, Sethi JS, Bignami A (1985) Glial fibrillary acidic (GFA) protein in vertebrates: immunofluorescence and immunoblotting study with monoclonal and polyclonal antibodies. *J Comp Neurol* 239:75-88.

Dahl D, Rueger DC, Bignami A, Weber K, Osborn M (1981) Vimentin, the 57 000 molecular weight protein of fibroblast filaments, is the major cytoskeletal component in immature glia. *Eur J Cell Biol* 24:191-196.

Das T, Payer B, Cayouette M, Harris WA (2003) In vivo time-lapse imaging of cell divisions during neurogenesis in the developing zebrafish retina. *Neuron* 37:597-609.

Doe CQ, Technau GM (1993) Identification and cell lineage of individual neural precursors in the Drosophila CNS. *Trends Neurosci* 16:510-514.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97:703-716.

Dorsky RI, Rapaport DH, Harris WA (1995) Xotch inhibits cell differentiation in the Xenopus retina. *Neuron* 14:487-496.

Drury RAB, and Wallington, EA (1967). Carleton's Histological Technique.

Durand B, Gao FB, Raff M (1997) Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation. *Embo J* 16:306-317.

Durand B, Raff M (2000) A cell-intrinsic timer that operates during oligodendrocyte development. *Bioessays* 22:64-71.

Echeverri K, Tanaka EM (2002) Ectoderm to mesoderm lineage switching during axolotl tail regeneration. *Science* 298:1993-1996.

Edlund T, Jessell TM (1999) Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. *Cell* 96:211-224.

Edmondson JC, Hatten ME (1987) Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. *J Neurosci* 7:1928-1934.

Eng LF, Ghirnikar RS, Lee YL (2000) Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). *Neurochem Res* 25:1439-1451.

Eng LF, Vanderhaeghen JJ, Bignami A, Gerstl B (1971) An acidic protein isolated from fibrous astrocytes. *Brain Res* 28:351-354.

Feng L, Hatten ME, Heintz N (1994) Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. *Neuron* 12:895-908.

Feng L, Heintz N (1995) Differentiating neurons activate transcription of the brain lipid-binding protein gene in radial glia through a novel regulatory element. *Development* 121:1719-1730.

Fischer AJ, Reh TA (2001) Muller glia are a potential source of neural regeneration in the postnatal chicken retina. *Nat Neurosci* 4:247-252.

Fischer AJ, Reh TA (2002) Exogenous growth factors stimulate the regeneration of ganglion cells in the chicken retina. *Dev Biol* 251:367-379.

Fischer AJ, Reh TA (2003) Potential of Muller glia to become neurogenic retinal progenitor cells. *Glia* 43:70-76.

Font E, Desfilis E, Perez-Canellas MM, Garcia-Verdugo JM (2001) Neurogenesis and neuronal regeneration in the adult reptilian brain. *Brain Behav Evol* 58:276-295.

Fraser S, Keynes R, Lumsden A (1990) Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* 344:431-435.

Fujita S (1963) The matrix cell and cytogenesis in the developing central nervous system. *J Comp Neurol* 120:37-42.

Furukawa T, Mukherjee S, Bao ZZ, Morrow EM, Cepko CL (2000) rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. *Neuron* 26:383-394.

Gahtan E, O'Malley DM (2003) Visually guided injection of identified reticulospinal neurons in zebrafish: a survey of spinal arborization patterns. *J Comp Neurol* 459:186-200.

Gaiano N, Nye JS, Fishell G (2000) Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* 26:395-404.

Galileo DS, Gray GE, Owens GC, Majors J, Sanes JR (1990) Neurons and glia arise from a common progenitor in chicken optic tectum: demonstration with two retroviruses and cell type-specific antibodies. *Proc Natl Acad Sci U S A* 87:458-462.

Gao FB, Durand B, Raff M (1997) Oligodendrocyte precursor cells count time but not cell divisions before differentiation. *Curr Biol* 7:152-155.

Garcia-Verdugo JM, Farinas I, Molowny A, Lopez-Garcia C (1986) Ultrastructure of putative migrating cells in the cerebral cortex of Lacerta galloti. *J Morphol* 189:189-197.

Garcia-Verdugo JM, Ferron S, Flames N, Collado L, Desfilis E, Font E (2002) The proliferative ventricular zone in adult vertebrates: a comparative study using reptiles, birds, and mammals. *Brain Res Bull* 57:765-775.

Gasser UE, Hatten ME (1990a) Neuron-glia interactions of rat hippocampal cells in vitro: glial-guided neuronal migration and neuronal regulation of glial differentiation. *J Neurosci* 10:1276-1285.

Gasser UE, Hatten ME (1990b) Central nervous system neurons migrate on astroglial fibers from heterotypic brain regions in vitro. *Proc Natl Acad Sci U S A* 87:4543-4547.

van Gehuchten, A (1894). Systeme nerveux des téléostéens. *La Cellule* 10, 256 – 291.

van Gehuchten, A (1898). La moelle epiniere des larves des Batraciens (Salamandra maculosa). *Archives de biologie* XV, 600 – 619.

Gilmour DT, Maischein HM, Nusslein-Volhard C (2002) Migration and function of a glial subtype in the vertebrate peripheral nervous system. *Neuron* 34:577-588.

Golden JA, Cepko CL (1996) Clones in the chick diencephalon contain multiple cell types and siblings are widely dispersed. *Development* 122:65-78.

Gongidi V, Ring C, Moody M, Brekken R, Sage EH, Rakic P, Anton ES (2004) SPARC-like 1 Regulates the Terminal Phase of Radial Glia-Guided Migration in the Cerebral Cortex. *Neuron* 41:57-69.

Götz M, Stoykova A, Gruss P (1998) Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron* 21:1031-1044.

Gray GE, Sanes JR (1992) Lineage of radial glia in the chicken optic tectum. *Development* 114:271-283.

Grove EA, Williams BP, Li DQ, Hajihosseini M, Friedrich A, Price J (1993) Multiple restricted lineages in the embryonic rat cerebral cortex. *Development* 117:553-561.

Haas K, Sin WC, Javaherian A, Li Z, Cline HT (2001). Single-cell electroporation for gene transfer in vivo. *Neuron* 29:583-591.

Hartfuss E, Galli R, Heins N, Gotz M (2001). Characterization of CNS precursor subtypes and radial glia. *Dev Biol* 229:15-30.

Heins N, Malatesta P, Cecconi F, Nakafuku M, Tucker KL, Hack MA, Chapouton P, Barde YA, Gotz M (2002) Glial cells generate neurons: the role of the transcription factor Pax6. *Nat Neurosci* 5:308-315.

Henrique D, Hirsinger E, Adam J, Le Roux I, Pourquie O, Ish-Horowicz D, Lewis J (1997) Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. *Curr Biol* 7:661-670.

Hinds JW, Ruffett TL (1971). Cell proliferation in the neural tube: an electron microscopic and golgi analysis in the mouse cerebral vesicle. *Z Zellforsch Mikrosk Anat* 115:226-264.

His, W. (1887). Die Entwickelung der ersten Nervenbahnen beim menschilchen Embryo. *Arch Anat Physiolo. Leipzig.* 92, 368 – 378.

Holder N, Clarke JD (1988) Is there a correlation between continuous neurogenesis and directed axon regeneration in the vertebrate nervous system? *Trends Neurosci* 11:94-99.

Holder N, Clarke JD, Kamalati T, Lane EB (1990) Heterogeneity in spinal radial glia demonstrated by intermediate filament expression and HRP labelling. *J Neurocytol* 19:915-928.

Holder N, Clarke JD, Stephens N, Wilson SW, Orsi C, Bloomer T, Tonge DA (1991) Continuous growth of the motor system in the axolotl. *J Comp Neurol* 303:534-550.

Holt CE, Bertsch TW, Ellis HM, Harris WA (1988) Cellular determination in the Xenopus retina is independent of lineage and birth date. *Neuron* 1:15-26.

Horstmann E (1954) The fiber glia of selacean brain. *Z Zellforsch Mikrosk Anat* 39:588-617.

Huttner WB, Brand M (1997) Asymmetric division and polarity of neuroepithelial cells. *Curr Opin Neurobiol* 7:29-39.

Ishibashi M, Moriyoshi K, Sasai Y, Shiota K, Nakanishi S, Kageyama R (1994) Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. *Embo J* 13:1799-1805.

Jacobson, M (1991). Developmental Neurobiology.

Jan YN, Jan LY (1994) Neuronal cell fate specification in Drosophila. *Curr Opin Neurobiol* 4:8-13.

Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J (1999) Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 96:25-34.

Kálmán M (1993) Vimentin persists in the mature glia of fish brain. *Neurobiology (Bp)* 1:47-54.

Kálmán M (1998) Astroglial architecture of the carp (Cyprinus carpio) brain as revealed by immunohistochemical staining against glial fibrillary acidic protein (GFAP). *Anat Embryol (Berl)* 198:409-433.

Kálmán M, Ari C (2002) Distribution of GFAP immunoreactive structures in the rhombencephalon of the sterlet (Acipenser ruthenus) and its evolutionary implication. *J Exp Zool* 293:395-406.

Kálmán M, Gould RM (2001) GFAP-immunopositive structures in spiny dogfish, Squalus acanthias, and little skate, Raia erinacea, brains: differences have evolutionary implications. *Anat Embryol (Berl)* 204:59-80.

Kálmán M, Kiss A, Majorossy K (1994) Distribution of glial fibrillary acidic protein-immunopositive structures in the brain of the red-eared freshwater turtle (Pseudemys scripta elegans). *Anat Embryol (Berl)* 189:421-434.

Kálmán M, Martin-Partido G, Hidalgo-Sanchez M, Majorossy K (1997) Distribution of glial fibrillary acidic protein-immunopositive structures in the developing brain of the turtle Mauremys leprosa. *Anat Embryol (Berl)* 196:47-65.

Kálmán M, Pritz MB (2001) Glial fibrillary acidic protein-immunopositive structures in the brain of a Crocodilian, Caiman crocodilus, and its bearing on the evolution of astroglia. *J Comp Neurol* 431:460-480.

Kamei Y, Inagaki N, Nishizawa M, Tsutsumi O, Taketani Y, Inagaki M (1998) Visualization of mitotic radial glial lineage cells in the developing rat brain by Cdc2 kinase-phosphorylated vimentin. *Glia* 23:191-199.

Kaufmann E, Knochel W (1996) Five years on the wings of fork head. *Mech Dev* 57:3-20.

Kawai H, Arata N, Nakayasu H (2001) Three-dimensional distribution of astrocytes in zebrafish spinal cord. *Glia* 36:406-413.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* 203:253-310.

Knoblich JA, Jan LY, Jan YN (1995) Asymmetric segregation of Numb and Prospero during cell division. *Nature* 377:624-627.

Köster RW, Fraser SE (2001) Direct imaging of in vivo neuronal migration in the developing cerebellum. *Curr Biol* 11:1858-1863.

Kumanishi T, Usui H, Ichikawa T, Nishiyama A, Katagiri T, Abe S, Yoshida Y, Washiyama K, Kuwano R, Sakimura K, et al. (1992) Human glial fibrillary acidic protein (GFAP): molecular cloning of the complete cDNA sequence and chromosomal localization (chromosome 17) of the GFAP gene. *Acta Neuropathol (Berl)* 83:569-578.

Laywell ED, Rakic P, Kukekov VG, Holland EC, Steindler DA (2000) Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc Natl Acad Sci U S A* 97:13883-13888.

Leber SM, Breedlove SM, Sanes JR (1990) Lineage, arrangement, and death of clonally related motoneurons in chick spinal cord. *J Neurosci* 10:2451-2462.

Lendahl U, Zimmerman LB, McKay RD (1990) CNS stem cells express a new class of intermediate filament protein. *Cell* 60:585-595.

von Lenhossék, M (1892). Beobachtungen an den Spinalganglion und dem Rueckenmark von Pristiurus-embryonen. *Anat.Anz.* 510 – 539.

Levine RL (1989) Organization of astrocytes in the visual pathways of the goldfish: an immunohistochemical study. *J Comp Neurol* 285:231-245.

Levitt P, Rakic P (1980) Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. *J Comp Neurol* 193:815-840.

Levitt P, Cooper ML, Rakic P (1981) Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: an ultrastructural immunoperoxidase analysis. *J Neurosci* 1:27-39

Lewis J (1998) Notch signalling and the control of cell fate choices in vertebrates. *Semin Cell Dev Biol* 9:583-589.

Li R, Messing A, Goldman JE, Brenner M (2002) GFAP mutations in Alexander disease. *Int J Dev Neurosci* 20:259-268.

Ling C, Zuo M, Alvarez-Buylla A, Cheng MF (1997) Neurogenesis in juvenile and adult ring doves. *J Comp Neurol* 379:300-312.

Lopez-Garcia C, Molowny A, Garcia-Verdugo JM, Ferrer I (1988) Delayed postnatal neurogenesis in the cerebral cortex of lizards. *Brain Res* 471:167-174.

Lopez-Garcia C, Molowny A, Garcia-Verdugo JM, Martinez-Guijarro FJ, Bernabeu A (1990) Late generated neurons in the medial cortex of adult lizards send axons that reach the Timm-reactive zones. *Brain Res Dev Brain Res* 57:249-254.

Lopez-Garcia C, Molowny A, Martinez-Guijarro FJ, Blasco-Ibanez JM, Luis de la Iglesia JA, Bernabeu A, Garcia-Verdugo JM (1992) Lesion and regeneration in the medial cerebral cortex of lizards. *Histol Histopathol* 7:725-746.

Lu B, Jan L, Jan YN (2000) Control of cell divisions in the nervous system: symmetry and asymmetry. *Annu Rev Neurosci* 23:531-556.

Luskin MB (1998) Neuroblasts of the postnatal mammalian forebrain: their phenotype and fate. *J Neurobiol* 36:221-233.

Luskin MB, Parnavelas JG, Barfield JA (1993) Neurons, astrocytes, and oligodendrocytes of the rat cerebral cortex originate from separate progenitor cells: an ultrastructural analysis of clonally related cells. *J Neurosci* 13:1730-1750.

Luskin MB, Pearlman AL, Sanes JR (1988) Cell lineage in the cerebral cortex of the mouse studied in vivo and in vitro with a recombinant retrovirus. *Neuron* 1:635-647.

Lyons DA, Guy AT, Clarke JD (2003) Monitoring neural progenitor fate through multiple rounds of division in an intact vertebrate brain. *Development* 130:3427-3436.

Mack AF, Fernald RD (1997) Cell movement and cell cycle dynamics in the retina of the adult teleost Haplochromis burtoni. *J Comp Neurol* 388:435-443.

Magini, G (1888) Ulteriori ricerche istologiche sul cervello fetale. *Rendiconti della R. Accademia dei Lincei* 4, 760-763.

Malatesta P, Hartfuss E, Götz M (2000) Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* 127:5253-5263.

Malatesta P, Hack MA, Hartfuss E, Kettenmann H, Klinkert W, Kirchhoff F, Götz M (2003) Neuronal or glial progeny: regional differences in radial glia fate. *Neuron* 37:751-764.

Manabe N, Hirai S, Imai F, Nakanishi H, Takai Y, Ohno S (2002) Association of ASIP/mPAR-3 with adherens junctions of mouse neuroepithelial cells. *Dev Dyn* 225:61-69.

Marcus RC, Delaney CL, Easter SS, Jr. (1999) Neurogenesis in the visual system of embryonic and adult zebrafish (Danio rerio). *Vis Neurosci* 16:417-424.

Marcus RC, Easter SS, Jr. (1995) Expression of glial fibrillary acidic protein and its relation to tract formation in embryonic zebrafish (Danio rerio). *J Comp Neurol* 359:365-381.

McNeely, J, Miller, K, and Reid, W (1990). Conserving the World's Biodiversity.

Memberg SP, Hall AK (1995) Dividing neuron precursors express neuron-specific tubulin. *J Neurobiol* 27:26-43.

Menezes JR, Smith CM, Nelson KC, Luskin MB (1995) The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. *Mol Cell Neurosci* 6:496-508.

Messing A, Brenner M (2003) GFAP: functional implications gleaned from studies of genetically engineered mice. *Glia* 43:87-90.

Messing A, Head MW, Galles K, Galbreath EJ, Goldman JE, Brenner M (1998) Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice. *Am J Pathol* 152:391-398.

Metcalfe WK, Mendelson B, Kimmel CB (1986) Segmental homologies among reticulospinal neurons in the hindbrain of the zebrafish larva. *J Comp Neurol* 251:147-159.

Meyer A, Biermann CH, Orti G (1993) The phylogenetic position of the zebrafish (Danio rerio), a model system in developmental biology: an invitation to the comparative method. *Proc R Soc Lond B Biol Sci* 252:231-236.

Miller RH, Liuzzi FJ (1986) Regional specialization of the radial glial cells of the adult frog spinal cord. *J Neurocytol* 15:187-196.

Mione MC, Cavanagh JF, Harris B, Parnavelas JG (1997) Cell fate specification and symmetrical/asymmetrical divisions in the developing cerebral cortex. *J Neurosci* 17:2018-2029.

Misson JP, Edwards MA, Yamamoto M, Caviness VS, Jr. (1988) Identification of radial glial cells within the developing murine central nervous system: studies based upon a new immunohistochemical marker. *Brain Res Dev Brain Res* 44:95-108.

Miyata T, Kawaguchi A, Okano H, Ogawa M (2001) Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* 31:727-741.

Morest DK (1970) A study of neurogenesis in the forebrain of opossum pouch young. *Z Anat Entwicklungsgesch* 130:265-305.

Nadarajah B, Brunstrom JE, Grutzendler J, Wong RO, Pearlman AL (2001) Two modes of radial migration in early development of the cerebral cortex. *Nat Neurosci* 4:143-150.

Nadarajah B, Alifragis P, Wong RO, Parnavelas JG (2002) Ventricle-directed migration in the developing cerebral cortex. *Nat Neurosci* 5:218-224.

Nadarajah B, Parnavelas JG (2002) Modes of neuronal migration in the developing cerebral cortex. *Nat Rev Neurosci* 3:423-432.

Nielsen AL, Jorgensen AL (2003) Structural and functional characterization of the zebrafish gene for glial fibrillary acidic protein, GFAP. *Gene* 310:123-132.

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409:714-720.

Noctor SC, Flint AC, Weissman TA, Wong WS, Clinton BK, Kriegstein AR (2002) Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J Neurosci* 22:3161-3173.

Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci* 7:136-144.

Nolte C, Matyash M, Pivneva T, Schipke CG, Ohlemeyer C, Hanisch UK, Kirchhoff F, Kettenmann H (2001) GFAP promoter-controlled EGFP-expressing transgenic mice: a tool to visualize astrocytes and astrogliosis in living brain tissue. *Glia* 33:72-86.

Nona SN, Shehab SA, Stafford CA, Cronly-Dillon JR (1989) Glial fibrillary acidic protein (GFAP) from goldfish: its localisation in visual pathway. *Glia* 2:189-200.

Nottebohm F, O'Loughlin B, Gould K, Yohay K, Alvarez-Buylla A (1994) The life span of new neurons in a song control nucleus of the adult canary brain depends on time of year when these cells are born. *Proc Natl Acad Sci U S A* 91:7849-7853.

O'Rourke NA, Dailey ME, Smith SJ, McConnell SK (1992) Diverse migratory pathways in the developing cerebral cortex. *Science* 258:299-302.

Odenthal J, Nusslein-Volhard C (1998) fork head domain genes in zebrafish. *Dev Genes Evol* 208:245-258.

Onteniente B, Kimura H, Maeda T (1983) Comparative study of the glial fibrillary acidic protein in vertebrates by PAP immunohistochemistry. *J Comp Neurol* 215:427-436.

Papan C, Campos-Ortega JA (1999) Region-specific cell clones in the developing spinal cord of the zebrafish. *Dev Genes Evol* 209:135-144.

Park HC, Kim CH, Bae YK, Yeo SY, Kim SH, Hong SK, Shin J, Yoo KW, Hibi M, Hirano T, Miki N, Chitnis AB, Huh TL (2000) Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. *Dev Biol* 227:279-293.

Parnavelas JG, Barfield JA, Franke E, Luskin MB (1991) Separate progenitor cells give rise to pyramidal and nonpyramidal neurons in the rat telencephalon. *Cereb Cortex* 1:463-468.

Patten BA, Peyrin JM, Weinmaster G, Corfas G (2003) Sequential signaling through Notch1 and erbB receptors mediates radial glia differentiation. *J Neurosci* 23:6132-6140.

Petersen PH, Zou K, Hwang JK, Jan YN, Zhong W (2002) Progenitor cell maintenance requires numb and numblike during mouse neurogenesis. *Nature* 419:929-934.

Pixley SK, de Vellis J (1984) Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. *Brain Res* 317:201-209.

Pouwels E (1978) On the development of the cerebellum of the trout, Salmo gairdneri. V. Neuroglial cells and their development. *Anat Embryol (Berl)* 153:67-83.

Price J (1987) Retroviruses and the study of cell lineage. *Development* 101:409-419.

Price J, Thurlow L (1988) Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. *Development* 104:473-482.

Price J, Williams B, Grove E (1991) Cell lineage in the cerebral cortex. *Development* Suppl 2:23-28.

Qian X, Shen Q, Goderie SK, He W, Capela A, Davis AA, Temple S (2000) Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* 28:69-80.

Rakic P (1971a) Guidance of neurons migrating to the fetal monkey neocortex. *Brain Res* 33:471-476.

Rakic P (1971b) Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in Macacus Rhesus. *J Comp Neurol* 141:283-312.

Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. *J Comp Neurol* 145:61-83.

Rakic P (1990) Principles of neural cell migration. *Experientia* 46:882-891.

Rakic P (2003) Elusive radial glial cells: historical and evolutionary perspective. *Glia* 43:19-32.

Ramón y Cajal, S (1911). Histologie du système nerveux de l'homme et des vertébrés. Paris: Maloine.

Reid CB, Tavazoie SF, Walsh CA (1997) Clonal dispersion and evidence for asymmetric cell division in ferret cortex. *Development* 124:2441-2450.

Retzius, G (1893). Studien über Ependym und Neuroglia bei Knochenfischen. In *Die nervösen Elemente im Rueckenmarke der Knochenfische.*

Rice DS, Curran T (2001) Role of the reelin signaling pathway in central nervous system development. *Annu Rev Neurosci* 24:1005-1039.

Roegiers F, Jan YN (2004) Asymmetric cell division. *Curr Opin Cell Biol* 16:195-205.

Rubio M, Suarez I, Bodega G, Fernandez B (1992) Glial fibrillary acidic protein and vimentin immunohistochemistry in the posterior rhombencephalon of the Iberian barb (Barbus comiza). *Neurosci Lett* 134:203-206.

Sancho-Tello M, Valles S, Montoliu C, Renau-Piqueras J, Guerri C (1995) Developmental pattern of GFAP and vimentin gene expression in rat brain and in radial glial cultures. *Glia* 15:157-166.

Sanes JR, Rubenstein JL, Nicolas JF (1986) Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *Embo J* 5:3133-3142.

Saper CB (1996) Any way you cut it: a new journal policy for the use of unbiased counting methods. *J Comp Neurol* 364:5.

Sauer, FC (1935a). Mitosis in the neural tube. *J Comp Neurol* 62,377-405.

Sauer, FC (1935b). The cellular structure of the neural tube. *J. Comp.Neurol.* 63, 13-23.

Scheer N, Groth A, Hans S, Campos-Ortega JA (2001) An instructive function for Notch in promoting gliogenesis in the zebrafish retina. *Development* 128:1099-1107.

Schmechel DE, Rakic P (1979a) A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes. *Anat Embryol (Berl)* 156:115-152.

Schmechel DE, Rakic P (1979b) Arrested proliferation of radial glial cells during midgestation in rhesus monkey. *Nature* 277:303-305.

Schmid RS, McGrath B, Berechid BE, Boyles B, Marchionni M, Sestan N, Anton ES (2003) Neuregulin 1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex. *Proc Natl Acad Sci U S A* 100:4251-4256.

Seymour RM, Berry M (1975) Scanning and transmission electron microscope studies of interkinetic nuclear migration in the cerebral vesicles of the rat. *J Comp Neurol* 160:105-125.

Shen Q, Zhong W, Jan YN, Temple S (2002) Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. *Development* 129:4843-4853.

Shen Q, Zhong W, Jan YN, Temple S (2002) Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. *Development* 129:4843-4853.

Shibata T, Yamada K, Watanabe M, Ikenaka K, Wada K, Tanaka K, Inoue Y (1997) Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. *J Neurosci* 17:9212-9219.

Shoukimas GM, Hinds JW (1978) The development of the cerebral cortex in the embryonic mouse: an electron microscopic serial section analysis. *J Comp Neurol* 179:795-830.

Silva AO, Ercole CE, McLoon SC (2002) Plane of cell cleavage and numb distribution during cell division relative to cell differentiation in the developing retina. *J Neurosci* 22:7518-7525.

Skoff RP, Price DL, Stocks A (1976) Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. II. Time of origin. *J Comp Neurol* 169:313-334.

Soula C, Foulquier F, Duprat AM, Cochard P (1993) Lineage analysis of early neural plate cells: cells with purely neuronal fate coexist with bipotential neuroglial progenitors. *Dev Biol* 159:196-207.

Sterio DC (1984) The unbiased estimation of number and sizes of arbitrary particles using the dissector. *J Microsc* 134:127-136.

Stensaas LJ, Stensaas SS (1968a) Astrocytic neuroglial cells, oligodendrocytes and microgliacytes in the spinal cord of the toad. I. Light microscopy. *Z Zellforsch Mikrosk Anat* 84:473-489.

Stensaas LJ, Stensaas SS (1968b) Light microscopy of glial cells in turtles and birds. *Z Zellforsch Mikrosk Anat* 91:315-340.

Stevenson JA, Yoon MG (1981) Mitosis of radial glial cells in the optic tectum of adult goldfish. *J Neurosci* 1:862-875.

Swarz JR, Oster-Granite ML (1978) Presence of radial glia in foetal mouse cerebellum. *J Neurocytol* 7:301-312.

Szele FG, Cepko CL (1996) A subset of clones in the chick telencephalon arranged in rostrocaudal arrays. *Curr Biol* 6:1685-1690.

Szele FG, Cepko CL (1998) The dispersion of clonally related cells in the developing chick telencephalon. *Dev Biol* 195:100-113.

Takahashi T, Nowakowski RS, Caviness VS, Jr. (1996) The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neuronogenesis. *J Neurosci* 16:6183-6196.

Tamamaki N, Nakamura K, Okamoto K, Kaneko T (2001) Radial glia is a progenitor of neocortical neurons in the developing cerebral cortex. *Neurosci Res* 41:51-60.

Tan SS, Breen S (1993) Radial mosaicism and tangential cell dispersion both contribute to mouse neocortical development. *Nature* 362:638-640.

Tomizawa K, Inoue Y, Nakayasu H (2000) A monoclonal antibody stains radial glia in the adult zebrafish (Danio rerio) CNS. *J Neurocytol* 29:119-128.

Toyoshima H, Hunter T (1994) p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* 78:67-74.

Tramontin AD, Garcia-Verdugo JM, Lim DA, Alvarez-Buylla A (2003) Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. *Cereb Cortex* 13:580-587.

Trevarrow B, Marks DL, Kimmel CB (1990) Organization of hindbrain segments in the zebrafish embryo. *Neuron* 4:669-679.

Turner DL, Cepko CL (1987) A common progenitor for neurons and glia persists in rat retina late in development. *Nature* 328:131-136.

Verdi JM, Schmandt R, Bashirullah A, Jacob S, Salvino R, Craig CG, Program AE, Lipshitz HD, McGlade CJ (1996) Mammalian NUMB is an evolutionarily conserved signaling adapter protein that specifies cell fate. *Curr Biol* 6:1134-1145.

Voigt T (1989) Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. *J Comp Neurol* 289:74-88.

Wakamatsu Y, Maynard TM, Jones SU, Weston JA (1999) NUMB localizes in the basal cortex of mitotic avian neuroepithelial cells and modulates neuronal differentiation by binding to NOTCH-1. *Neuron* 23:71-81.

Walsh C, Cepko CL (1993) Clonal dispersion in proliferative layers of developing cerebral cortex. *Nature* 362:632-635.

Wang RT, Halpern M (1982a) Neurogenesis in the vomeronasal epithelium of adult garter snakes. 1. Degeneration of bipolar neurons and proliferation of undifferentiated cells following experimental vomeronasal axotomy. *Brain Res* 237:23-39.

Wang RT, Halpern M (1982b) Neurogenesis in the vomeronasal epithelium of adult garter snakes. 2. Reconstitution of the bipolar neuron layer following experimental vomeronasal axotomy. *Brain Res* 237:41-59.

Wasowicz M, Ward R, Reperant J (1999) An investigation of astroglial morphology in torpedo and scyliorhinus. *J Neurocytol* 28:639-653.
Weissman T, Noctor SC, Clinton BK, Honig LS, Kriegstein AR (2003) Neurogenic radial glial cells in reptile, rodent and human: from mitosis to migration. *Cereb Cortex* 13:550-559.

Westerfield, M (1995). The Zebrafish Book.

Wetts R, Fraser SE (1988) Multipotent precursors can give rise to all major cell types of the frog retina. *Science* 239:1142-1145.

Wicht H, Derouiche A, Korf HW (1994) An immunocytochemical investigation of glial morphology in the Pacific hagfish: radial and astrocyte-like glia have the same phylogenetic age. *J Neurocytol* 23:565-576.

Williams BP, Price J (1995) Evidence for multiple precursor cell types in the embryonic rat cerebral cortex. *Neuron* 14:1181-1188.

Wilson SW, Ross LS, Parrett T, Easter SS Jr (1990) The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, Brachydanio rerio. *Development* 108:121-145.

Wodarz A, Huttner WB (2003) Asymmetric cell division during neurogenesis in Drosophila and vertebrates. *Mech Dev* 120:1297-1309.

Zamora AJ (1978) The ependymal and glial configuration in the spinal cord of urodeles. *Anat Embryol (Berl)* 154:67-82.

Zhong W (2003) Diversifying neural cells through order of birth and asymmetry of division. *Neuron* 37:11-14.

Zhong W, Feder JN, Jiang MM, Jan LY, Jan YN (1996) Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. *Neuron* 17:43-53.

Zhong W, Jiang MM, Schonemann MD, Meneses JJ, Pedersen RA, Jan LY, Jan YN (2000) Mouse numb is an essential gene involved in cortical neurogenesis. *Proc Natl Acad Sci U S A* 97:6844-6849.

Zhong W, Jiang MM, Weinmaster G, Jan LY, Jan YN (1997) Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. *Development* 124:1887-1897.

Zhuo L, Sun B, Zhang CL, Fine A, Chiu SY, Messing A (1997) Live astrocytes visualized by green fluorescent protein in transgenic mice. *Dev Biol* 187:36-42.

Zhuo L, Theis M, Alvarez-Maya I, Brenner M, Willecke K, Messing A (2001) hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. *Genesis* 31:85-94.

Zilian O, Saner C, Hagedorn L, Lee HY, Sauberli E, Suter U, Sommer L, Aguet M (2001) Multiple roles of mouse Numb in tuning developmental cell fates. *Curr Biol* 11:494-501.

Zimmerman RP, Polley EH, Fortney RL (1988) Cell birthdays and rate of differentiation of ganglion and horizontal cells of the developing cat's retina. *J Comp Neurol* 274:77-90.

Zupanc GK (1999) Neurogenesis, cell death and regeneration in the adult gymnotiform brain. *J Exp Biol* 202 (Pt 10):1435-1446.

Zupanc GK (2001) Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish. *Brain Behav Evol* 58:250-275.

Zupanc GK, Clint SC (2003). Potential role of radial glia in adult neurogenesis of teleost fish. *Glia* 43:77-86.

Zupanc GK, Ott R (1999). Cell proliferation after lesions in the cerebellum of adult teleost fish: time course, origin, and type of new cells produced. *Exp Neurol* 160:78-87.

Zupanc GK, Zupanc MM (1992). Birth and migration of neurons in the central posterior/prepacemaker nucleus during adulthood in weakly electric knifefish (Eigenmannia sp.). *Proc Natl Acad Sci U S A* 89:9539-9543.